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(54) Title: NOVEL PEPTIDE ANTIGENS AND IMMUNOASSAYS, TEST KITS AND VACCINES USING THE SAME

(57) Abstract

A peptide having specific immunoreactivity to antibodies to HTLV-I, HTLV-II, or combinations thereof comprising a peptide selected from the group consisting of Env-1 (HTLV-I; a.a 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT, and analogues thereof, wherein the amino acids in the sequence may be substituted as long as the immunoreactivity to antibodies to HTLV-I or HTLV-II derived from the three dimensional conformation of the sequences are substantially preserved. An immunoassay method for the detection of antibodies to HTLV-I, HTLV-II or a combination thereof, a test kit for the detection of the antibodies, a peptide composition containing the peptides and a vaccine.

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NOVEL PEPTIDE ANTIGENS AND IMMUNOASSAYS, TEST KITS AND VACCINES USING THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to peptides derived from structural gene products of HTLV-I and HTLV-II selected from the group consisting of Env-1 (HTLV-I; amino acids (a.a.) 191-215), Env-2 (HTLV-II; a.a. 187-210), Env5 (HTLV-I; a.a. 242-257); Gagla (HTLV-I; a.a. 102-117), Pol-3 (HTLV-I; a.a. 487-502), Env-20 (HTLV-II; a.a. 85-102), Env-23 (HTLV-II; a.a. 274-289), Gag-10 (HTLV-I/II; a.a. 364-385) and Ers (endogenous retroviral sequence) and immunoassays, test kits and vaccines using these peptides.

Discussion of Related Art

Human T-cell lymphotropic viruses (HTLV) types I and II are closely related human retroviruses (Wachsman W, Golde DW, Chen ISY. HTLV and human leukemia: Perspectives. Semin Hematol 1986;23:246-56). HTLV-I is etiologically associated with adult T-cell leukemia (ATL) and with a chronic neurologic disorder known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP; (Ehrlich GD, Poiesz BJ. Clinical and molecular parameters of HTLV-I infection. Clin Lab Med 1988;8:65-84). In contrast, HTLV-II, which was first isolated from a patient with a variant of hairy cell leukemia (Kalyanaraman VS, Sarngadharan MG, Robert-Guroff M, et al. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. Science 1982;218:571-3), has not been associated with any specific disease (Blattner WA. Retroviruses. In: Evans AS, ed. Viral Infections of Humans: Epidemiology and Control, ed 3. New York: Plenum 1989:545-92). While HTLV-I infection is endemic in southwestern Japan, the Caribbean, and some regions of Africa (Ehrlich GD, Poiesz BJ. Clinical and molecular parameters of HTLV-I infection. Clin Lab Med 1988;8:65-84), HTLV-II has been reported mainly in intravenous drug users (Lee H, Swanson P, Shorty VS, Zack JA, Rosenblalt JD, Chen I. High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. Science

1989;244:471-5.) Concern about transmission of HTLV-I/II infection from contaminated blood products has been intensified by serologic evidence of HTLV-I in volunteer blood donors (Williams AE, Fang CT, Slamon DJ, et al. Seroprevalence and epidemiological correlation of HTLV-I infection in U.S. blood donors. Science 1988;240:643-6; Anderson D.W., Epstein J.S., Lee T.H., et al. Serologic confirmation of human T-lymphotropic virus type I infection in healthy blood and plasma donors. Blood 1989;74:2585-91), and the U.S. Food and Drug Administration has suggested HTLV-I screening of all donated blood (Public Health Service working group. Licensure of screening tests for antibody to human T-lymphotropic virus type-I. MMWR 1988;37:736-47; Kaplan J.E., Khabbaz R.F. HTLV-I: Newest addition to blood donor screening. Am. Fam. Physician 1989;40:189-95). Recent data from the screening of blood donors indicate that more than half of those seropositive for HTLV-I indeed may be infected with HTLV-II (Chen ISY, Rosenblat JD, Black AC, Arrigo SJ, Green PL. 1990. HTLV-II Prevalence and regulation of gene expression. AIDS Res Hum Retroviruses 6:134-5.) In addition, a high percentage of the HTLV seroreactivity among intravenous drug users in the United States may be due to HTLV-II infection (Lee H, Swanson P, Shorty V.S., Zack J.A., Rosenblalt J.D., Chen I. High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. Science 1989;244:471-5). In the absence of serological assays that can distinguish HTLV-I from HTLV-II infection (Chen I.S.Y., Rosenblat J.D., Black A.C., Arrigo S.J., Green P.L. 1990. HTLV-II Prevalence and regulation of gene expression. AIDS Res. Hum. Retroviruses 6:134-5), counselling such individuals about HTLV-I associated diseases may be inappropriate.

The overall structural similarity as well as the identity of much of the primary amino acid sequence (Myers G, Josephs S.F., Rabson A.B., Smith T.F., Wong Staal F. In: Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.M. 1988) would suggest antigenic

cross-reactivity between HTLV-I and HTLV-II, and indeed, none of the serological assays, to date, can reliably distinguish between these two infections (Anderson D.W., Epstein J.S., Lee T.H., et al. Serologic confirmation of human T-lymphotropic virus type I infection in healthy blood and plasma donors. Blood 1989;74:2585-91; Lee T.H., Coligan J.E., McLane M.F., et al. Serologic cross-reactivity between envelope gene products of type I and type II human T-cell leukemia virus. Proc. Natl. Acad. Sci. USA 1984;81:7579). While virus isolation and gene amplification techniques (Lee H, Swanson P, Shorty VS, Zack JA, Rosenblalt JD, Chen I. High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. Science 1989;244:471-5; De B, Srinivasan A. Detection of human immunodeficiency virus (HIV) and human lymphotropic virus type I or II dual infections by polymerase chain reaction. Oncogene 1989;4:1533-5) can differentiate HTLV-I from HTLV-II infection, these methods are labor intensive and require collection and processing of lymphocytes. A serologic assay that could distinguish the two infections is highly desirable. Such an assay would be very useful both for seroepidemiologic studies that have thus far been hampered by the inability to distinguish the two viruses and for the purpose of counseling blood donors and others who test seropositive (Chen I.S.Y., Rosenblat J.D., Black A.C., Arrigo S.J., Green P.L. 1990. HTLV-II Prevalence and regulation of gene expression. AIDS Res. Hum. Retroviruses 6:134-5).

Synthetic peptides representing conserved "immuno-dominant" epitopes provide an attractive alternative to virus-derived antigens in view of their low cost and ability to be accurately reproduced. The analysis of antibodies reactive with predetermined amino acid sequences (Lerner R.A. Antibodies of predetermined specificity in biology and medicine. Adv. Immunol. 1984;36:1-44) has been shown previously to be both a sensitive and specific means to distinguish related

retrovirus infections from each other (Norrby E, Biberfeld G, Chiodi F, et al. Discrimination between antibodies to HIV and to related retroviruses using site directed serology. Nature 1987;329:248-50; Gnann J.W., McCormick J.B., Mitchell S., Nelson J.A., Oldstone MBA. 1987. Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. Science 237:1346-9). Because structural proteins such as env, gag and pol from both HTLV-I and HTLV-II are major immunodominant proteins under conditions of natural infection (Lee T, Coligan J.E., Homma T, McLane M.F., Tachibana N, Essex M. Human T-cell leukemia virus-associated membrane antigens (HTLV-MA): Identity of the major antigens recognized following virus infection. Proc. Natl. Acad. Sci. USA 1984;81:3856-60; Kanner S.B., Mayer C.C., Geffin R.B., et al. Human retroviral env and gag polypetides; Serologic assays to measure infection. J. Immunol. 1986;137:674-8), the present inventors have analyzed the serologic reactivity of those regions of the env, gag and pol of HTLV-I and HTLV-II that exhibited considerable differences in the amino acid sequences (Sodroski J., Patarca R., Perkins D., et al. Sequence of the Envelope glycoprotein gene of Type II human T-lymphotropic virus. Science 1984;225:421-4).

U.S. Patent 4,833,701 discloses a peptide composition having specific immunoreactivity to antibodies to HTLV-I.

SUMMARY OF THE INVENTION

One object of the present invention is to define a major immunodominant epitope of the HTLV proteins that does not show cross-reactivity with serum specimens from HTLV-II-infected individuals.

Thus, one embodiment of the present invention relates to a peptide having specific immunoreactivity to antibodies to HTLV-I, HTLV-II, or combinations thereof comprising a peptide selected from the group consisting of:

Env-1 (HTLV-I; a.a 191-215)LPHSNLDHILEPSIPWKSKLLTLV,

Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI,
Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY,
Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI,
Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK,
Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP,
Env-23 (HTLV-II; a.a. 274-289)QPRLQAITTDNCNNSI,
Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP,
Ers (endogenous retroviral sequence)
PRIPPKPCPICVCPNWKSDCPT, and

analogues thereof, wherein the amino acids in the sequence may be substituted as long as the immunoreactivity to antibodies to HTLV-I or HTLV-II derived from the three dimensional conformation of the sequences are substantially preserved.

The invention is further directed to an immunoassay method for the detection of antibodies to HTLV-I, HTLV-II or a combination thereof, a test kit for the detection of said antibodies, a peptide composition containing said peptides and a vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the location of synthetic peptide in HTLV-1 genome upper panel and HTLV-II (lower panel). The relative position of each peptide is shown by the box.

Figures 2A and 2B show antibodies to purified HTLV-I protein (upper panel) or Env-5 peptide (lower panel) in patients with HTLV-I infection (HTLV-I), with HTLV-I infections that have been confirmed by PCR (HTLV-I PCR) and with HTLV-II infection that have been PCR confirmed (HTLV-II PCR). The shaded area represents the mean + 3 SD of the responses of 21 normal persons.

Figure 3 shows the competition by Env-5 (•-•), HTLV-I (o-o) or HTLV-II (Δ-Δ) purified proteins of anti-Env-5 antibodies in HTLV-I infected individuals. Serial 1:2 dilutions of a 10 ug/ml peptide or HTLV proteins solution are mixed 1:1 with a 1:10 dilution of test serum. The mixtures are allowed to incubate overnight at 4°C. Each is then assayed for anti-Env-5 activity by ELISA. The results are expressed as the mean percentage inhibition of four HTLV-I infected sera.

Figures 4A and 4B show IgG antibodies to Gag 1a (upper panel) or Pol-3 (lower panel) peptide in patients with HTLV-I infection (o), HTLV-II (□) infection and normal controls (△). The shaded symbol in the HTLV-I infected group represents antibody responsiveness of the individuals with HAM/TSP or ATL.

Figures 5A and 5B show a computer prediction of the secondary structure of gag encoded protein of HTLV-I (Top) and HTLV-II (bottom) superimposed with the value for antigenic index. The radius of a circle over a residue is proportional to the mean antigenic index as calculated for the residue plus the next five residues. The parameters for hydrophilicity, flexibility and surface probability are averaged over five amino acid residues, with a limit of 0.7 for hydrophilicity, 1.04 for flexibility and 5.0 for surface probability.

Figure 6 shows the alignment of Env-20^{85-102}, Env-$202^{173-209}$, and Env-$203^{219-256}$ with corresponding HTLV-I sequences. Identical amino acid residues between HTLV-II and HTLV-I are in boxes. Amino acid residue numbering is from the N-terminus of each protein.

Figure 7 shows antibodies to Env-20^{85-102}, Env-$202^{173-209}$, and Env-$203^{219-256}$ in serum specimens from blood donors infected with HTLV-II (HT-II) and HTLV-I (HT-I). Shaded area represents means +2SD of the response of 22 healthy blood donors.

Figures 8A and 8B show seroreactivity of $HTLV^{pos}$ and $HTLV^{ind}$ specimens with synthetic peptides from env(A) and gag (B) region of HTLV and an endogenous retroviral sequence (B). Data is expressed as percent reactivity of $HTLV^{pos}$ (p19&/orp24+&gp46/61; n=30) and $HTLV^{ind}$ (p19+p24+r21+, n=12; p19+p24+, n=10; p19+, n=43; p24+, n=6, r21+, n=9) specimens. A: % reactivity for synthetic HTLV-I specific Env-$1^{191-213}$ (▣) and Env-$5^{242-256}$ (▨); HTLV-II specific Env-$2^{187-209}$ (▨) and Env-20^{85-102} (□); B: % reactivity with HTLV-I specific Gag-$1a^{102-117}$ (▣), HTLV-I/II specific Gag-$10^{364-385}$ (▨), and an endogenous retroviral sequence

RTVL^{EIA} (▨).

Figure 9 shows the RTVL region contains two imperfect copies of a conserved sequence in a location similar to that found in other retroviruses. The period in Figure 9 represents a gap in the sequence which has been added for sequence alignment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a highly sensitive method for the detection of antibodies to HTLV-I or HTLV-II in body fluids by the use of synthetic peptides. The peptides are also useful as a vaccine by stimulating the production of antibodies of HTLV-I or HTLV-II to provide protection against infection by HTLV-I or HTLV-II in healthy mammals, including humans. The peptides have amino acid sequences which correspond to segments on the envelope protein and are highly immunoreactive with antibodies in sera of patients infected with HTLV-I or HTLV-II. The detection method includes an enzyme-linked immunosorbent assay (ELISA), an immunoradiometric assay (IRMA), and other forms of immunoassay procedures such as enzyme immuno blotting assay on nitrocellulose paper and hemagglutination assay using the peptides as the antigen.

An immunoassay for HTLV needs to be developed that satisfy two main criteria. A test must distinguish HTLV-I and HTLV-II in locales where both viruses are endemic. Enzyme immuno assays (EIA) where whole virus lysates are used as a source of antigens cannot effectively distinguish HTLV-I from HTLV-II due to the sequence homology in highly conserved regions of the core and the polymerase protein of these viruses. Two immunoassays must be available to laboratories involved in blood screening that are highly sensitive and specific. In the present study, the inventor reports that synthetic peptides from immunoreactive domains of HTLV-I and HTLV-II viral proteins offer an approach to design an immunoassay that will distinguish HTLV-I from HTLV-II. The inventor also provides evidence that the synthetic peptide derived from the polymerase region of HTLV-I detects serum antibodies

in most infected individuals.

Abbreviations for amino acids used herein are conventionally defined as described hereinbelow.

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

According to the present invention, peptides useful for the detection of antibodies to HTLV-I or HTLV-II are selected from the group consisting of:

Env-1 (HTLV-I; a.a 191-215)LPHSNLDHILEPSIPWKSKLLTLV,
Env-2 (HTLV-II, a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI,
Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY,
Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI,
Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK,
Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP,
Env-23 (HTLV-II; a.a. 274-289)QPRLQAITTDNCNNSI,
Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP,
Ers (endogenous retroviral sequence)
PRIPPKPCPICVCPNWKSDCPT, and

analogues thereof, wherein the amino acids in the sequence may be substituted as long as the immunoreactivity to antibodies to HTLV-I or HTLV-II derived from the three dimensional conformation of the sequences are substan-

tially preserved.

These peptides may comprise analogues or segments, i.e., a shorter or longer peptide chain by having more amino acids added to the terminal amino acids of the above sequence or having a few less of the terminal amino acids from either terminal. It is expected that as long as the three dimensional conformation recognizable by the dominant antibodies to HTLV-I or HTLV-II is preserved, analogues of the synthetic peptides may also comprise substitution and/or deletion of the recited amino acids of the above sequences.

Based on the high degree of sensitivity and specificity of the peptides according to the present invention in the immunoreaction of antibodies to HTLV-I or HTLV-II, it is believed that the peptides may also be useful as a vaccine (e.g., for ATL and HAM/TSP) and as immunogens for the development of both monoclonal and polyclonal antibodies to HTLV-I and HTLV-II in mammals, including humans. The peptides when coupled to a protein or a polymer carrier or when polymerized to homo or hetero dimers or high oligomers by cysteine oxidation, induced disulfide cross linking, or when polymerized to homo- or heterodimers or higher oligomers by use of homo- or heterofunctional multivalent cross linking reagents, can be introduced to normal subjects to stimulate production of antibodies to HTLV-I or HTLV-II and provide protection against infection in healthy mammals. Since the peptides according to the present invention are not derived biochemically from the virus, there is no danger of exposing the normal subjects who are to be vaccinated to the disease.

The advantages of using the peptides according to the present invention are many. The peptides are chemically synthesized. This means that there is no involvement with the HTLV-I or HTLV-II virus at any time during the process of making the test reagent or the vaccine. During the preparation of the vaccine or the vaccination process, there is no risk of exposure of the

production workers or individuals in the health profession to the HTLV-I or HTLV-II virus. Similarly, there is no risk or exposure to HTLV-I or HTLV-II in the use of these peptides or the development of monoclonal or polyclonal antibodies to HTLV-I or HTLV-II in mammals. Further, up to the final step of the test to detect antibodies to HTLV-I or HTLV-II, where the test reagent is exposed to samples of sera or body fluid, there is no risk of exposure of the laboratory worker to the HTLV-I or HTLV-II virus. Any risk of exposure in this final step can be further avoided by taking the precautionary step of heating the serum samples, which are to be tested, at 60°C for half an hour, thereby deactivating the virus.

Another problem which is avoided by the present invention is the possibility of false positive results caused by the presence in antigenic materials from host cells co-purified with the HTLV-I or HTLV-II viral preparation or E. coli derived proteins co-purified with expressed viral fragments. Certain normal individuals have antibodies to E. coli or human leukocyte antigens, e.g., HLA, which are cross reactive with the antigenic materials from host cells. Sera samples from these normal individuals even though they have not been exposed to HTLV-I or HTLV-II, may show a positive response in the ELISA or IRMA tests.

A diagnosis that a person may be infected with HTLV-I or HTLV-II based on this type of false positive response can bring severe anxiety to the person and his/her family. All of these problems can be avoided by using the peptides of the present invention as the test reagents.

Further, with appropriate amino acid analogue substitutions, it is expected that various peptide analogues based on the prescribed amino acid sequence can be synthesized with properties giving rise to lower background readings or better adsorption capacity to solid phases useful for HTLV-I or HTLV-II antibody screening assays.

Moreover, because the peptides of the present invention are synthetically prepared, the quality can be controlled and as a result, reproducibility of the test results can be assured. Also, since very small amounts of peptides are required from each test procedure, and because the expense of preparing the peptide is relatively low, the cost of screening body fluids for antibodies to HTLV-I or HTLV-II and the preparation of a vaccine is relatively low.

A method of detecting antibodies to HTLV-I, HTLV-II or combinations thereof in body fluids comprises preparing at least one of the above-mentioned peptides, analogues, or a mixture thereof, and using about 0.1 mg to about 20 mg, preferably about 1.0 mg to about 10 mg per test in a buffer at a pH of about 7.5 to 10, preferably about 9.4 to 9.8, of at least one peptide as the antigen in an immunoassay procedure.

The peptide prepared in accordance with the present invention can be used to detect HTLV-I and HTLV-II infection by using it as the test reagent in any form of immunoassay such as an enzyme-linked immunoadsorbent assay (ELISA), an enzyme immunodot assay, a hemagglutination assay, a radioimmunoradiometric assay (IRMA), or any variety of competitive binding assays.

The present invention is further directed to an immunoassay method for the detection of antibodies to HTLV-I, HTLV-II or combinations thereof which comprises: (i) coating a solid support or other labeling material with an effective amount of a peptide of the invention for reacting with antibodies to HTLV-I, HTLV-II or combinations thereof in an amount sufficient to produce an antibody-peptide complex to be detected, (ii) adding a test sera diluted with a buffer wherein the antibodies to HTLV-I or HTLV-II in the test sera form a peptide-antibody complex with said peptide, (iii) incubating the mixture, and (iv) detecting the presence of the peptide-antibody complex. In step (iv), a second known antibody labelled with an enzyme and a substrate is introduced which reacts

with the enzyme to form a colored product. Also, in step (iv), a second known antibody labelled with a radioactive element is introduced. Alternatively, in step (iv), the peptide antibody complex may also be detected by agglutination. The solid support may be further coated with at least one of the peptides in the invention in a multidot array. The amount of the peptide is preferably in the range of 1 mg to 10 mg per dot. The detection step (iv) may also be done competitively using labeled or unlabeled antigen or antibody to compete with the complex. The antigen of step (i) need not be attached in any way provided that the antibody-antigen complex may be detected such as by polyethylene glycol precipitation or by a Coombs reagent.

The invention is also directed to a test kit for the detection of antibodies to HTLV-I, HTLV-II, or combinations thereof, which comprises: a solid support or other suitable labeling material having attached thereto; an immunoadsorbent comprising at least one peptide of the invention or simply said at least one peptide alone; a sample of normal serum as a negative control; a sample of serum containing antibodies of HTLV-I or HTLV-II as a positive control, and a buffer for diluting the serum samples.

Furthermore, the invention is directed to a peptide composition comprising at least one of the peptides of the invention. When more than one peptide is present in the composition, each is present in a ratio of 1:1 with respect to one another. For instance, when several peptides are present in the mixture, they are in a ratio of 1:1:1. Each peptide may be preferably present in an amount of 0.5 mg to 5 mg. An example of two more peptides to be mixed together includes a combination of Env-1 and Env-5. This mixture of peptides may provide for an increase in sensitivity for HTLV-I detection. Similarly, Env-2 and Env-20 may be combined in order to increase HTLV-II detection. If necessary, the peptides may be mixed in a suitable carrier such as saline or a

mixture of phosphate buffer in saline.

The invention is also directed to a vaccine containing at least one of the peptides of the invention and is used to generate antibodies and other cells and products of the immune response. Any of the peptides alone or in combination may be conjugated to conventionally known carrier proteins and animals may be immunized prior to infection with HTLV-I/II. The peptides generating high immune response (both B- and T-cell responses) may be used to develop the vaccine in a conventional manner.

Env-5 (HTLV-I; amino acids 242-257) is the most immunodominant epitope and reacts with all of the serum specimens from patients infected with HTLV-I with no cross-reaction from 35 persons infected with HTLV-II. Even though the number of samples tested in this study are small, they represent HTLV-I/II-infected patients from various clinical groups as well as various geographic disease-endemic areas, and based on current estimated HTLV-I/II seroprevalence rate in blood donors (0.02%), the number for HTLV-I-positive subjects (n = 52) would represent the number of similarly infected persons predicted for a population of 260,000.

The envelope protein of HTLV-I is known to show variability for different viral isolates (Daenke S., Nightingale S., Cruickshank J.K., Bangham C.R.M. Sequence variants of human T-Cell lymphotropic virus type I from patients with Tropical Spastic Paraparesis and adult T-cell leukemia do not distinguish neurological from leukemia isolates. J. Virol. 1990;64:1278-82), and this could effect the sensitivity of the test. A comparison of the amino acid sequences of the Env-5 region (SerProAsnValSerValProSerSerSerSerThrProLeu- LeuTyr) with other viral isolates reveals that 13 of 16 (81%) amino acids are conserved. Preliminary data on epitope mapping of Env-5 suggest that the critical part of the epitope is not found at the three amino acid positions (amino acids 247, 250, and 251) that show variability.

The Env-1 peptide (HTLV-I; amino acids 191-215) demonstrates a high sensitivity for HTLV-I infection (92%), but a small percentage of HTLV-II infected subjects (8.6%) also reacts with this peptide, probably reflecting some degree of structural homology. Env-2 (HTLV-II; amino acids 187-210), on the other hand, reacts with both HTLV-I (94%) and HTLV-II (77%) serum samples. Thus, even though the peptide is chosen from a region within the HTLV-II sequence that has considerable differences in the amino acid sequence from the HTLV-I sequence, evidently, the epitope is mimicked in such a way that it is recognized by antibodies in both HTLV-I and HTLV-II infected serum specimens and could be included in future peptide assays for serologic determination of HTLV-I/II infection.

Other investigators have used recombinant proteins (Samuel KP, Lautenberger J.A., Jorcyk C.L., Joseph S., Wong-Staal F., Papas T.S.. Diagnostic potential for human malignancies of bacterially produced HTLV-I envelope protein. Science 1984; 226:1094-7; Tachibana N., Miyoshi I., Papas T.S., Essex M. Antibody reactivity to different regions of human T-cell leukemia virus Type I gp61 in infected people. J. Virol. 1989;63:4952-7) or synthetic peptide technology to identify antigenic sites on the envelope (Palker T.J., Tanner M.E., Scearce R.M., Streilein R.D., Clark M.E., Haynes B.F. Mapping of immunogenic regions of human T-cell leukemia virus Type I (HTLV-I) gp46 and gp21 envelope glycoproteins with Env - encoded synthetic peptides and a monoclonal antibody to gp46. J. Immunol. 1989; 142:971-8; Copeland T.D., Tsai W.P., Kim Y.D., Oroszlan S. Envelope proteins of human T-cell leukemia virus type-I: characterization of antisera to synthetic peptides and identification of a natural epitope. J. Immunol. 1986;137:2945-51) proteins of HTLV-I. For example, one of our peptides (Env-1, amino acids 191-215) overlaps with a region of gp46 (amino acids 190-209) that contains both a T- and B-cell epitope (Palker T.J., Tanner M.E., Scearce R.M., Streilein R.D., Clark M.E., Haynes B.F. Mapping of immunogenic regions of human

T-cell leukemia virus Type I (HTLV-I) gp46 and gp21 envelope glycoproteins with Env - encoded synthetic peptides and a monoclonal antibody to gp46. J. Immunol. 1989;142:971-8; Copeland T.D., Tsai W.P., Kim Y.D., Oroszlan S. Envelope proteins of human T-cell leukemia virus type-I: characterization of antisera to synthetic peptides and identification of a natural epitope. J. Immunol. 1986;137:2945-51; Kurata A, Palker T.J., Streilein R.D., Scearce R.M., Haynes B.F., Berzofsky J.A. Immunodominant sites of human T-cell lymphotropic virus Type I envelope protein for murine helper T-cells. J. Immunol. 1989;143:2024-30). More recently, a recombinant fusion protein (MTA-4; 42 amino acids) reactive with a human monoclonal has been shown to specifically react only with HTLV-I infected serum samples (Foung S.K.H., Lipka J.J., Bui K. Determination of a unique and immunodominant epitope of HTLV-I. Presented at the 3rd Annual Conference of Retrovirology, Hawaii, 1990. (Abs)). The epitope of this monoclonal antibody has been mapped to amino acids 185-196 (Ralston S., Hoeprich P., Akita R. Identification and synthesis of the epitope for a human monoclonal antibody which can neutralize human T-cell leukemia/lymphotropic virus type I. J. Biol. Chem. 1989;264:16343-6.), which overlaps with our Env-1 peptide. Plasmids containing sequences corresponding to the carboxyterminal region of HTLV-I gp46 plasmid pKS 300, amino acids 200-306 (Samuel K.P., Lautenberger J.A., Jorcyk C.L., Joseph S., Wong-Staal F., Papas TS. Diagnostic potential for human malignancies of bacterially produced HTLV-I envelope protein. Science 1984;226:1094-7) and plasmid RP-C, amino acids 229-308 (Tachibana N, Miyoshi I, Papas TS, Essex M. Antibody reactivity to different regions of human T-cell leukemia virus Type I gp61 in infected people. J. Virol. 1989;63:4952-7) are recognized by antibodies from HTLV-I infected persons. Interestingly, the Env-5 synthetic peptide (amino acids 242-257) is contained within the regions encoded by the plasmids. These studies and the data presented here

confirm that the C-terminal region of gp46 is highly immunogenic in humans. Of greater importance is the finding that serum specimens from HTLV-II-infected persons do not react with this epitope, thus defining an antigenic determinant at the C-terminal region of HTLV-I gp46 that is not shared by HTLV-II.

Thus, the Env-5 peptide-based assay provides a simplified, inexpensive, highly sensitive, and extremely specific test for discrimination of HTLV-I from HTLV-II infection, and could, therefore, easily replace the PCR procedure now used to distinguish the two viruses. The findings that the inventor can achieve enhanced diagnostic specificity by a peptide-based ELISA is supported by earlier reports demonstrating serologic discrimination of HIV-1 and HIV-2 infection (Norrby E., Biberfeld G., Chiodi F., et al. Discrimination between antibodies to HIV and to related retroviruses using site directed serology. Nature, 1987;329:248-50; Gnann J.W., McCormick J.B., Mitchell S., Nelson J.A., Oldstone M.B.A. 1987. Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. Science 237:1346-9). Although the finding of specific immunodominant epitope(s) of HTLV-I has obvious diagnostic implications, it is also important to recognize the potential role this epitope may have in inducing T-cell proliferation or virus neutralizing antibodies.

The proteins encoded by the gag, pol, and env gene of HTLV contributes to many of the pathological and functional properties of the virus which may be relevant during the course of the infection and the progression of the disease (Hopp T.P., Woods K.R. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci., USA 1981;78:3824-8). Using a series of synthetic peptides with predicted antigenic epitopes from conserved amino-acid regions, the present inventor has tried to locate structural motifs within HTLV-I and HTLV-II for B-cell specific antibody recognition. Of the various peptides derived from the gag and pol region of

both HTLV-I and HTLV-II, only two react with serum specimens from HTLV-I/II infected individuals. Gag-1a defined from the C-terminal of p19 protein (HTLV-I; aa 102-117) is the most immunodominant epitope and reacts with 90% of the HTLV-I infected sera subjects; a small percentage of HTLV-II infected sera also react with this peptide (11%), reflecting some degree of antigenic homology within the HTLV-I and HTLV-II.

Furthermore, serum antibody reactivity against Gag 1a can be specifically inhibited with HTLV-I which represents a conformationally "native" epitope present on the HTLV-I. Therefore, the Gag 1a peptide with amino acid sequence Pro Pro Ser Ser Pro Thr His Asp Pro Pro Asp Ser Asp Pro Gln Ile represents an immunodominant domain of HTLV-I that is recognized by serum antibodies from most of HTLV-I infected persons. Moreover, the Gag1a based immunoassay allows a serologic distinction between the closely related HTLV-I and HTLV-II infection.

The immunodominance of the C-terminal region of p19 gag protein ties together with two other findings. Palker et al (Palker, T. J., Scearce, R., M., Copeland, T. D., Oroszlan, S., and Haynes, B. F., 1986. C-terminal region of human T-cell lymphotropic virus type I (HTLV-I) p19 core protein is immunogenic in humans and contains an HTLV-I specific epitope. J. Immunol., 136, 2393-2397) have described an epitope at the C-terminus of p19 down stream from our Gag 1a which react with 6 of the 8 serum specimen tested. In their study, only 2 of 8 specimens show reactivity to the epitope which overlaps with Gag 1a and can be due to the sensitivity of the test system used in their study. More recently, Kurodata et al (Kuroda, N., Washitani, Y., Shiraki, H., Kiyokawa, H., Ohno, M., Sato, H., and Maeda, Y., 1990. Detection of antibodies to human T-lyphotropic virus type I by using synthetic peptides. Int. J. Cancer, 45, 865-868) have reported an immunodominant epitope contained within the amino acids 100-130 at the C-terminus of p19 which reacts with 100% HTLV-I infected serum specimen. The specificity of this

peptide for HTLV-II infected serum specimens is not tested in their study. These studies and the data presented here confirm that the C-terminal region of p19 is highly immunogenic and further demonstrates that the C-terminus of p19 contains a strain specific epitope which is HTLV-I specific.

In natural infection with a retrovirus, the host generally makes antibodies to the products of the gag or env gene or both (Schupbach, J., Kalynaraman, J., Sarngatiaran, G., Blattner, W., and Gallo, R., 1983. Antibodies against three purified proteins of human types C retroviruses, human T-cell leukemia-lymphoma virus, in adult T-cell leukemia-lymphoma patients and healthy blacks from the Caribbean, Cancer Res 43, 886-891.; Gallo, D., Hoffman, M, N., Lossen, C, K., Diggs, J, L., Hurst, J, W., and Penning, L, M., 1988. Comparison of immunofluorescence, Enzyme immunoassay and western blot (immunoblot) methods for detection of antibody to Human T-cell leukemia virus Type-I, J. Clin Microbiol., 26, 1487-1491). Antibody responses to the products of the pol gene during HTLV-I/II infection have not been described. The results presented here demonstrate that the majority of individuals infected with the HTLV-I and HTLV-II have readily detectable levels of antibodies to the peptide derived from the central region of the pol gene. The results presented here clearly indicate that similar to antibody responsiveness to pol gene products during HIV infection, HTLV-I pol products also induce antibody responses. The antisera raised to this peptide would help to evaluate the structural characteristic of pol products.

EXAMPLE 1

Methods

A total of 186 serum specimens from various study groups are chosen for this study (Table 1).

Table 1.

Standard serologic results and PCR data of study population

Group	No. tested	Serology HTLV-I/II	PCR Confirmation Group HIV-1	HTLV-I	HTLV-II
HTLV-I					
U.S. residents*	20	+	-	+	-
Japanese	32	+	-	ND**	ND
HTLV-II					
U.S. residents	35	+	-	-	+
HIV-1					
U.S. residents	28	-	+	ND	ND
Other***	50	-	ND	ND	ND
Normal	21	-	-	ND	ND

* Two of the persons within the groups were of Caribbean origin.

** ND - not determined

*** Serum from patients with non retroviral infection.

The sera includes 87 specimens from subjects who are seropositive to HTLV-I/II. With the exception of 32 specimens kindly provided by Dr. M. Osame, Kagoshima, Japan, all of the serum specimens are determined to be from HTLV-I or HTLV-II-positive persons by polymerase chain reaction (PCR) assays (De B., Srinivasan A. Detection of human immunodeficiency virus (HIV) and human lymphotropic virus type I or II dual infections by polymerase chain reaction. Oncogene 1989; 4:1533-5), using peripheral blood lymphocytes from these same persons. Of these 55 PCR-confirmed specimens, 20 are from persons infected with HTLV-I whereas the other 35 are from persons infected with HTLV-II. Of the 20 HTLV-I-infected subjects, 13 have either ATL or HAM/TSP syndrome, and the other 7 are asymptomatic blood donors. The HTLV-II-

infected subjects are mostly intravenous drug users. Twenty serum specimens within this group are obtained from commercial sources (Serologics, Inc., Marietta, Ga.).

For comparison, serum specimens from 28 patients with confirmed human immunodeficiency virus (HIV) infection manifesting as asymptomatic (n = 10) or acquired immunodeficiency syndrome (AIDS) (n = 18) are tested. Serum specimens from patients with a variety of other clinical diseases (n = 50) are used to test for non-specific interference (Anderson D.W., Epstein J.S., Lee T.H., et al. Serologic confirmation of human T-lymphotropic virus type I infection in healthy blood and plasma donors. Blood 1989;74:2585-91). These specimens include those with rheumatoid factor (n = 3), nuclear antibodies (n = 3) and anti - HLA - DR antibodies (n = 1), those with viral infection (cytomegalovirus, n = 3; Epstein-Barr virus, n = 3; herpes simplex virus, n = 3; hepatitis B virus, n = 4; and rubella virus, n = 3) and those with parasitic infection (Plasmodium falciparum, n = 3; Toxoplasma gondii, n = 3; Trypanosoma cruzi, n = 5; Schistosoma mansoni, n = 5; Strongyloides stercoralis, n = 6; and Wuchereria bancrofti, n = 5). Serum specimens from 21 normal blood donors serve as a negative control.

Reference HTLV and HIV Antibody Tests

Patients serum specimens are initially tested for HTLV-I antibodies with a commercial enzyme-linked immunosorbent assay (HTLV-I ELISA, Dupont, Wilmington, Del.), according to the manufacturer's recommendations. Specimens that are repeatedly reactive are further tested by Western blotting and radioimmunoprecipitation assay as described previously (Hartley T.M., Khabbaz R.F., Cannon R.O., Kaplan J.E., Lairmore M.D. Characterization of antibody reactivity to human T-cell lymphotropic virus types I/II using immunoblot and radioimmunoprecipitation assays. J. Clin. Microbiol. 1990;28:646-50). Briefly, purified HTLV-I antigen (MT-2 cell line, Miyoshi I., Kubonishi I., Yoshimoto S., et al. Type C virus particles in a cord T-cell line derived by co-cultivating normal

human cord leukocytes and human leukemia T-cells. Nature 1981;296:770-3) obtained from Hillcrest Biologicals, Cypress, Calif., is diluted in sodium dodecyl sulfate sample buffer (0.125 M Tris HCl, pH 6.8, 5% 2ME, 4% SDS), boiled for 3 min and electrophoresed in a 10% polyacrylamide gel with a 3% stacking gel. The separated proteins are electroblotted onto nitrocellulose paper. Individual strips are incubated with 1:100 dilution of serum, washed, and incubated for 1 h with 5 mg of biotinylated goat anti-human (heavy- and light-chain) immunoglobulin G (Vector Laboratories, Burlingame, Calif.) per ml. Following reaction with an avidin-biotin-horseradish peroxidase conjugate and further washing, immune reactions are visualized with diaminobenzidine-nickel chloride-hydrogen peroxide as a substrate. For radioimmunoprecipitation assay, MT-2 cell lines are metabolically labeled (200 mCi of each amino acid/ 10^7 cells/ ml) with [^{35}S] cysteine and [^{35}S] methionine (New England Nuclear, Boston, Mass.). The labeled cells are washed in phosphate-buffered saline (PBS) and extracted in PBS containing 0.1% SDS and 0.02% Triton X-100. The detergent solubilized proteins are reacted with serum specimens, immune complexes precipitated by Protein-A Sepharose (Sigma, St. Louis, Mo.), run on a 10% polyacrylamide gel followed by autoradiography of the dried gel (Hartley TM, Khabbaz R.F., Cannon R.O., Kaplan J.E., Lairmore M.D. Characterization of antibody reactivity to human T-cell lymphotropic virus types I/II using immunoblot and radioimmunoprecipitation assays. J. Clin. Microbiol. 1990;28:646-50). A serum specimen is determined to be HTLV-I/II-positive if antibody reactivity is detected to at least two different HTLV structural gene products (gag p24 and env gp46 and/or gp68) either by Western blotting or RIPA analysis. Serum specimens reacting with only gag or env gene products are considered indeterminate and are not included in this study.

The antibodies to HIV proteins are determined by both ELISA and Western blot (Dupont), and only those

specimens having antibodies to both gag and env proteins are included.

Polymerase-Chain-Reaction Assays

Polymerase chain reaction (PCR) is performed with total genomic DNA isolated from patients peripheral blood lymphocytes by using reaction conditions as described previously (Saiki R., Gelfand, Stoffel S., et al. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487-9). Oligonucleotide primer pairs from pol and gag genes of HTLV-I and HTLV-II are used to amplify 1 mg total genomic DNA for each PCR amplification (De B, Srinivasan A. Detection of human immunodeficiency virus (HIV) and human lymphotropic virus type I or II dual infections by polymerase chain reaction. Oncogene 1989;4:1533-5.; De B., Srinivasan A. Multiple primer pairs for the detection of HTLV-I by PCR. Nucleic Acids Res. 1989; 17:2142). The amplified products are analyzed on a 5.0% polyacrylamide gel and confirmed further by Southern blot hybridization, using specific pol and gag nucleotide ^{32}P labeled probes. Genomic DNA preparation from MT-2 cells (HTLV-I), MO-T (HTLV-II), and Hut-78 (uninfected) are used as controls. A sample is defined as HTLV-I- or HTLV-II-positive based on its reactivity with primer pairs in two separate gene products.

Peptide Selection and Synthesis

Using published amino acid sequences (Myers G, Josephs S.F., Rabson A.B., Smith T.F., Wong Staal F. In: Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.M. 1988), the inventors aligned HTLV-I and HTLV-II sequences in their envelope regions. Four peptides are selected for synthesis by identifying regions in which HTLV-I and HTLV-II shows considerable amino acid differences (Fig 1). A cysteine residue is added to the N-terminus of each peptide to facilitate conjugation with proteins for studies not reported here. Secondary structure characteristics of the envelope protein are predicted (Chou P.Y., Fasman G.D. Prediction of the secondary structure of proteins from their amino

acid sequence. Adv Enzymol 1978;47:45) by entering amino acid sequences into the "Pepplot" program (M. Gribskov, University of Wisconsin, Madison Wisc.), and hydrophilicity characteristics are calculated by the method of Hopp and Woods (Hopp T.P., Woods K.R. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA, 1981;78:3824-8).

Synthetic peptides are made on the MilliGen 9050 Pepsynthesizer with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, using the manufacturer's reagents and recommended chemistry cycles. Peptides are cleaved from the resin, precipitated, and extracted several times with anhydrous ether. Final purification is by preparative high performance liquid chromatography (HPLC) on a Waters C18 Delta-Pak (19 mm x 30 cm, 15u particle, 300u pore size), using 0.1% trifluoroacetic acid (TFA) in water as the starting solvent followed by a 0-50% acetonitrile gradient in 0.1% TFA. Amino acid composition, amino acid sequence analysis, and analytical reverse phase HPLC are performed to confirm peptide sequence and purity.

Quantitative Assessment of Antibodies to Synthetic Peptides

Polyvinyl plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, VA.) are coated with 50 ul of synthetic peptides (100 ug/ml) in 0.01M carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates are washed with PBS containing 0.05% Tween-20 (PBS-T) six times, and each well is incubated with 200 ml of 3% bovine serum albumin (BSA) in PBS-T for 1 h at 37°C to block excess reactive sites. After the wells are washed, a 1:20 dilution of each test serum is added to duplicate wells and the plates are incubated for 90 min at 37°C and rinsed with PBS-T. Alkaline phosphatase conjugated, goat anti human IgG (Sigma, St. Louis, Mo.) is added and incubated for 90 min at room temperature, followed by addition of p-nitrophenyl phosphate (Sigma) substrate. The plates are read with an ELISA reader (SLT Lab Instrument, Austria) at

405 nm. Each serum specimen is also assayed in plates coated with BSA or unrelated synthetic peptide to control for nonspecific antibody binding. Seropositivity is defined as any value greater than the mean of the normal controls + 3 standard deviations.

Competitive Inhibition Assay

Inhibition of antibody binding to the synthetic peptide is carried out by adding increasing concentrations of synthetic peptide or purified HTLV-I or HTLV-II antigen (1-10 mg/ml) in the ELISA. The serum is mixed with the inhibition antigen immediately before it is added to the Env-5 peptide-coated plate, followed by assay as described above. The results are expressed as the percentage inhibition of antibody binding.

Statistical Analysis

Student's t-test is used for statistical evaluation as noted.

RESULTS

Quantitation of Human Antibodies to Synthetic Peptide

A non-competitive enzyme linked immunosorbent assay (ELISA) is developed using synthetic peptides (Env-1, Env-2 and Env-5) as a solid phase to detect strain-specific antibodies in HTLV-I/II infected subjects. In a series of experiments (data not shown), the inter-assay and intra-assay coefficient of variation (CV) are less than 15% and less than 8%, respectively. When this assay configuration is used to detect antibodies in a panel of serum specimens from patients infected with HTLV-I or HTLV-II, the highest percentage (100%) of seropositivity in the HTLV-I group is seen for Env-5, Env1, Env2, Gagla and Pol-3.

Table-2

Reactivity of serum specimens to synthetic peptides.

Group	No. tested	No. Positive*				
		Env-1	Env-2	Env-5	Gagla	Pol-3
HTLV-I	52	48(92)**	49(94)	52(100)	50(96)	50(96)
HTLV-II	35	3(8.5)	27(77)	0(0)	6(11.5)	34(88)
HIV-1	28	0(0)	ND***	0(0)	0(0)	0(0)
Other Infection	34	0(0)	ND	0(0)	0(0)	0(0)
Controls	21	0(0)	0(0)	0(0)	0(0)	3(14)

* Positive value defined by O.D higher than the mean +3 S.D of 21 normal controls.

** Number in parenthesis is percent positive.

*** ND - not determined.

None of the 35 HTLV-II-infected serum specimens react with Env-5.

Env-1 demonstrates a high degree of reactivity (48/52; 92%) with serum specimens from HTLV-I infected persons and some cross-reaction (3/35; 8.6%) with specimens from HTLV-II-infected persons. Env-2, although derived from HTLV-II sequence, reacts strongly with serum specimens from both HTLV-I (49/52; 94%) and HTLV-II (27/35; 77%) infected persons. Of the 21 serum specimens from normal controls and the 78 specimens from subjects with other infections, including HIV, none react with any of these peptides.

One of the synthetic peptides termed Gag 1a (HTLV-I; a.a. 102-117) derived from the Gag encoded protein of HTLV-I, demonstrates a high degree of reactivity (47/52;90%) with serum specimens from HTLV-I infected persons and some cross reaction (4/35;11%) with pecimens from HTLV-II infected persons (Table-2). Of the six peptides derived from the pol encoded gene proteins, only pol-3 (HTLV-I; a.a. 487-502) react with both HTLV-I (50/52; 96%) and HTLV-II (30/35; 86%) infected serum specimens.

Specificity of antibodies detected by Env-5

Serologic cross-reactivity of serum specimens from

HTLV-I-and HTLV-II-infected patients is well documented (Anderson D.W., Epstein J.S., Lee T.H., et al. Serologic confirmation of human T-lymphotropic virus type I infection in healthy blood and plasma donors. Blood 1989; 74:2585-91.; Lee T.H., Coligan J.E., McLane M.F., et al. Serologic cross-reactivity between envelope gene products of type I and type II human T-cell leukemia virus. Proc. Natl. Acad. Sci. USA, 1984; 81:7579). Since the present inventors observed a high degree of sensitivity (100% for HTLV-I) and specificity (no cross-reaction with HTLV-II) with the Env-5 based assay, they wanted to confirm that the HTLV-II-infected study population indeed contains antibodies that cross-react in standard serologic assays which utilize HTLV-I viral antigen. All 35 serum specimens from HTLV-II-infected subjects have levels of antibody to HTLV-I (>3 SD above the mean for 21 normal controls) and there is no significant difference in the antibody levels between these HTLV-I-infected subjects and persons infected with HTLV-II ($P > .05$) (Fig. 2). When these specimens from HTLV-II-infected subjects are tested in the Env-5 based immunoassay, the cross-reactivity seen in the serologic assay for HTLV-I is no longer observed (Fig-2). This markedly enhanced specificity of the Env-5 assay has absolutely no effect on the diagnostic sensitivity of the test. Among the 32 serum specimens from HTLV-I-infected asymptomatic Japanese patients and 20 serum specimens from HTLV-I infected persons (confirmed by PCR), all are positive in the Env-5 assay. Further, there is no statistically significant difference ($P > .05$) in the antibody levels to Env-5 between Japanese asymptomatic patients and HTLV-I-infected, PCR-confirmed patients.

To further demonstrate the specificity of the Env-5, the present inventors next perform a competitive inhibition experiment with serum specimens from four HTLV-I infected patients by preincubating the serum specimens with Env-5 peptide, and HTLV-I and HTLV-II antigen. The antibody reactivity against Env-5 could be specifically inhibited by preincubating the serum specimen with Env-5

peptide or HTLV-I protein in a dose dependent manner, whereas incubation with a HTLV-II protein or unrelated peptide does not show any inhibition (Fig. 3).

Distribution of antibodies to Gag 1a and Pol-3

To evaluate the relative distribution of antibodies, serum levels of antibodies to peptides are compared in HTLV-I infected (asymptomatic and those with HAM/TSP), HTLV-II infected (asymptomatic) and normal controls. Most of the reactivity for Gag 1a antibodies is found in the HTLV-I infected group (Fig-4). Within this group, there is no significant difference in the levels of antibodies to Gag 1a in those who are asymptomatic and those with HAM/TSP ($p>.05$). In contrast, seroreactivity of Pol-3 was significantly higher in patients with HAM/TSP when compared with HTLV-I asymptomatic individuals (Fig-4). A significant proportion of serum specimens from HTLV-II reacts with this peptide and the levels of antibodies are similar to levels in HTLV-I infected asymptomatic individuals. In addition, four of the 35 serum specimens from normal donors demonstrate low levels of reactivity to Pol-3.

Antigenic index characteristics of Gag 1a and Pol-3

The secondary structure characteristics of gag and pol protein of both HTLV-I and HTLV-II are analyzed by using computer algorithms developed by Chou and Fasman (Chou, P. Y., Fasman, G. D., 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol., 47, 47). Figure 5 shows a secondary structure prediction for the gag region of HTLV-I and HTLV-II. Superimposed on the structural backbone are domains of high antigenic indices. The antigenic index is an algorithm designed by Jameson and Wolf to predict surface domains for combined values of flexibility, hydrophilicity and (Chou, P. Y., and Fasman, G. D., 1974. Prediction of Protein Confirmation. Biochemistry, 13, 222-244). One of the four regions with high antigenic indices lie within the Gag 1a domain. The other three antigenic determinants are located near the C-

terminus (amino acid nos. 337-342; 390-395 and 402-408). The three highest antigen index domains within HTLV-II gag are located at the amino acid position 343-348; 403-408 and 405-411. The absence of such a structural motif within the HTLV-II sequence (Figs. 5A and 5B) most likely is responsible for the lack of antibody responsiveness to this peptide in sera from individuals infected with HTLV-II. A similar analysis of pol protein of HTLV-I demonstrates Pol-3 to be located in an area of high hydrophilicity and antigenic index. In contrast, although other peptides such as Polla and Pol-4 demonstrate high antigen index, less than 10% of sera from infected individuals react with these peptides (data not shown).

In the following examples, 0.25% by weight of glutaraldehyde may be added in the coating buffer to facilitate better peptide binding on the plates or beads. Further, horseradish peroxidase conjugated mouse monoclonal anti-human IgG antibody may be used in place of horseradish peroxidase conjugated goat anti human IgG (Fc) as the second antibody tracer.

The gelatin used in these processes can include calf skin gelatin, pig skin gelatin, fish gelatin or any known available gelatin proteins or be replaced with albumin proteins.

EXAMPLE 2

Detection of Antibodies to HTLV-I or HTLV-II by an Enzyme-Linked Immunoadsorbent Assay.

Wells of 96-well plates are coated at 4°C overnight (or 3 hours at room temperature), with at least one of the peptides of the invention at 1.5 mg per well of the mixture in 100 ml 10 mM $NaHCO_3$ buffer, pH 9.5. The wells are washed three times with phosphate buffered saline (PBS) and then incubated with 250 ul of 3% by weight of gelatin in PBS at 37°C for 1 hour to block non-specific protein binding sites, followed by three more washes with PBS containing 0.05% by volume of Tween 20. The test sera (blood taken from a human patient or normal individual) are diluted with PBS containing 20% by volume normal goat

serum, 1% by weight gelatin and 0.05% by volume Tween 20 at dilutions of 1:20 and 1:200, volume to volume, respectively. 200 ml of the diluted sera are added to each well and allowed to react for 1 hour at 37°C. The wells are washed three times with 0.05% by volume Tween 20 in PBS in order to remove unbound antibodies. Horseradish peroxidase conjugated goat anti-human IgG (Fc) is used as a second antibody tracer to bind with the HTLV-I or HTLV-II antibody-antigen complex formed in positive wells. 100 ml of peroxidase labeled goat anti-human IgG at a dilution of 1:3000 in 1% by volume normal goat serum, 0.05% by volume Tween 20 in PBS is added to each well and incubated at 37°C for another 15 minutes.

The wells are washed five times with 0.05% by volume Tween 20 in PBS to remove unbound antibody and reacted with 100 μl of the substrate mixture containing 0.04% by weight orthophenylenediamine (OPD) and 0.012% by volume hydrogen peroxide in sodium citrate buffer, pH 5.0. This substrate mixture is used to detect the peroxidase label by forming a colored product. Reactions are stopped by the addition of 100 ml of 1.0M H_2SO_4 and the absorbance measured using an ELISA reader at 492 nm (i.e., A_{492}). Assays are performed in duplicate with one dilution (1:20) of serum samples from normal individuals or from patients with diseases unrelated to HTLV-I or HTLV-II infection used as negative controls. Absorbance readings greater than the cutoff value of A_{492}=0.12, (about 3 x the mean A_{492} value of normal serum control are taken as positive).

EXAMPLE 3

The procedure of Example 2 is repeated using the same sera samples as in Example 2 except that the well plates are precoated with 1 mg per well heat inactivated NP40 solubilized HTLV-I.

EXAMPLE 4

Detection of Antibodies of HTLV-I or HTLV-II by an Immunoradiometric Assay (IRMA)

Wells of 96-well flexible-polyvinylchloride (PV) plates are coated at 4°C overnight (or 3 hours at room

temperature) with at least one of the peptides of the invention at 1.5 mg per well in 100 ml 10mM $NaHCO_3$ buffer, pH 9.5. The wells are washed three times with phosphate buffered saline (PBS) and then incubated with 250 ul of 3% by weight gelatin in PBS at 37°C for 1 hour to block the non-specific protein binding sites, followed by three or more washes with PBS containing 0.05% by volume Tween 20. The test sera (blood taken from a human patient or normal individual) are diluted with PBS containing 20% by volume normal goat serum, 1% by weight gelatin and 0.05% by volume Tween 20 at dilutions of 1:20 and 1:2000 (volume to volume) respectively. 200 ml of the diluted sera are added to each well and allowed to react for 1 hour at 37°C. The wells are then washed three times with 0.05% by volume Tween 20 in PBS in order to remove unbound antibodies. I-125 labeled affinity purified goat anti-human IgG(Fc) is used as a second antibody tracer that binds with the antibody-antigen complex formed in positive wells. 100 ul of I-125 labeled goat antihuman IgG of 50,000-200,000 cpm in 1% by volume normal goat serum, 0.05% by volume Tween 20 in PBS is added to each well and incubated at 37°C for another hour.

The wells are washed five times with 0.05% to volume Tween-20 in PBS to remove unbound second antibody and dried. The wells are cut and counted by a gamma-scintillation counter. Assays are performed in duplicate with a 1:20 dilution volume to volume. Normal sera sample as negative controls are also tested simultaneously. Cpm readings greater than the average reading of normal sera samples + 4SD (standard deviation) are taken as positive.

EXAMPLE 5

Detection of Antibodies to HTLV-I or HTLV-II by a Hemagglutination Assay using at least one of the peptides of the invention coated gelatin articles, Erythrocytes of different animal species or latex beads as the solid phase immunoadsorbent.

One ml thoroughly washed erythrocytes, gelatin particles, polystyrene latex beads are coated with at

least one of the peptides of the invention at concentrations in the range of 5 mg/ml to 1 mg/ml. The peptide mixture coated cells, particles or beads are then incubated with serially diluted serum samples in the wells of a 96-well U-shaped microplate. After being left at room temperature for about an hour, the agglutination patterns on the bottom are read and the largest dilution showing a positive reaction is recorded.

This is a one-step assay which could be used for both qualitative and quantitative analysis of the presence of antibodies to HTLV-I or HTLV-II in specimens including sera or biofluids.

EXAMPLE 6

A third test kit for detecting HTLV-I or HTLV-II antibodies using the hemagglutination assay comprises a compartmented enclosure containing multiple 96-well U-shaped microplates and materials or hemagglutination assay including (1) a bottle containing erythrocytes, gelatin particles or latex polystyrene beads coated with at least one of the peptides of the invention; (2) normal human serum (as a negative control); and (3) heat inactivated, seropositive HTLV-I or HTLV-II serum (as a positive control). The procedure described in Example 2 is to be followed.

EXAMPLE 7

A diagnostic test kit for HTLV-I or HTLV-II antibody detection can be constructed. The test kit comprises a compartmented enclosure containing multiple 96-well plates coated prior to use with 1.5 m per well of at least one peptide of the present invention in 100 ml pH 9.5 10mM $NaHCO_3$ buffer. The kit further comprises materials for enzyme detection in separate sealed containers of: (1) normal human serum (as negative control); (2) heat inactivated HTLV-I or HTLV-II seropositive serum (as positive control); (3) normal goat serum; (4) peroxidase labeled-goat antihuman IgG; and (5) a color change indicator of orthophenylenediamine (OPD) and hydrogen peroxide in phosphate citrate buffer. The

procedure described in Example 2 is to be followed.

In this test, 96-well plates, precoated with the peptide of the present invention, can be replaced by polystyrene beads, or multiple mini-columns filled with controlled pore size glass beads, or nitrocellulose paper strip pre-coated with the peptides of the present invention for use as the solid phase immunoadsorbent.

EXAMPLE 8

A second test kit for detecting antibodies using the immunoradiometric assay (IRMA) comprises a compartmented enclosure containing multiple 96-well bendable polyvinylchloride (PVC) plates precoated with at least one peptide according to the present invention at a concentration of 1.5 mg per well of the peptide in 100 ml of pH 9.5 10mM $NaHCO_3$ buffer and materials for radioimmunoassay including: (1) normal human serum (as negative control); (2) heat inactivated, seropositive HTLV-I or HTLV-II serum (as positive control); (3) normal goat serum; and (4) I-125 labeled goat anti human IgG. The procedure described in Example 4 is to be followed.

In this test kit, 96-well PVC plates precoated with the peptides of the present invention can be replaced by polystyrene beads precoated with the peptide of the present invention for use as the solid phase immunoadsorbent.

Infection with human T cell lymphotropic virus type I (HTLV-I) and type II (HTLV-II) has been detected worldwide (Manns and Blattner, Transfusion, 31:67-75, 1991). HTLV-I has been associated with adult T cell leukemia (ATL) and HTLV-I-associated myelopathy (HAM), but HTLV-II has not been conclusively associated with any specific disease (CDC, MMWR, 39, 915, 921-924, 1990; Hjelle et al., J. Infect. Dis., 163:435-440, 1991), although an association with hairy cell leukemia was originally suggested (Kalyanaraman et al., Science, 218:571-573, 1982; Rosenblatt et al., N. Engl. J. Med., 313:372-377, 1986). Lack of serologic tests that can easily distinguish between HTLV-I and HTLV-II have made it

difficult to appropriately counsel those who test seropositive for HTLV and to establish clinical correlates of HTLV-II infection.

Molecular methods such as polymerase chain reaction (PCR) amplification have recently been used to unequivocally differentiate between HTLV-I and HTLV-II (Kwok et al., J. Infect. Dis., 158:1193-1197, 1988; Lee et al., Science, 244:471-475, 1989). Studies using these methods have defined intravenous (IV) drug users as the highest risk group for HTLV-II infection (Lee et al., Science, 244:471-475, 1989; Varnier et al., JAMA, 265, 597, 1991). Analysis of HTLV seropositive blood donors within the United States has further demonstrated that about half of the infected individuals are infected with HTLV-II (CDC, MMWR, 39, 915, 921-924, 1990; Sandler et al., Yale J. Biol. Med., 63:353-360, 1990; Hjelle et al., J. Infect. Dis., 163, 435-440, 1990). In addition, HTLV-II infection has recently been shown to be endemic in Guaymi Indians in Panama (Lairmore et al, Proc. Natl. Acad. Sci. USA, 87:8840-8844, 1990; Heneien et al., N. Engl. J. Med., 324, 565, 1991), Navajo and Pueblo tribes in New Mexico (Hjelle et al., J. Infect. Dis., 163, 435-440, 1991), and Seminole Indians in Florida (Levine PH et al., Unpublished), suggesting that HTLV-II infection has probably been endemic in American Indians for many years.

While humoral immune responses to structural proteins encoded by the gag, pol, and env genes of HTLV-I have been well characterized (Palker et al., J. Immunol. 142, 971-978, 1989; Chen et al., J. Virol., 63, 4952-4957, 1989; Horal et al., Human Retrovirology, HTLV (W.A. Blattner Ed.) p. 461-467, Plenum Press, NY, 1990; Lipka et al., J. Infect. Dis., 162:353-357, 1990; Lal et al., J. Infect. Dis., 163, 41-46, 1991; J. Virol., 65:1870-1876, 1991), little is known about the antibody responses to HTLV-II proteins. Two immunodominant epitopes of HTLV-II^{env} identified by synthetic peptide Env-2$^{187-210}$ (Lal et al., J. Infect. Dis., 163, 41-46, 1991) or recombinant protein RP-IIB$^{96-235}$ (Chen et al., Lancet, 336, 1153-1155, 1990) have

demonstrated predominant reactivity with antibodies in persons infected with both HTLV-I and HTLV-II. To further identify the structural motifs of the HTLV-II envelope glycoprotein (Sodroski et al., Science, 225:421-424, 1984), peptides of various lengths were synthesized spanning the HTLV-II^{env} glycoprotein and identified linear antigenic determinants recognized by antibodies from patients infected with HTLV-II.

More specifically, a series of synthetic peptides derived from the envelope glycoprotein of human T lymphotropic virus type II (HTLV-II) were used in an enzyme immunoassay to determine the immunodominant epitopes of envelope glycoprotein. Of the ten synthetic peptides spanning the external glycoprotein of HTLV-II (gp52) and four from the transmembrane protein (gp21), three peptides from gp52 (termed Env-20$^{85-102}$, Env-202$^{173-209}$, and Env-203$^{219-256}$) reacted with most of the PCR confirmed HTLV-II specimens (83%, 95%, and 76%, respectively); all other peptides reacted minimally with these specimens. Env-202$^{173-209}$ reacted with greater percentage (91% to 100%) of specimens from different risk groups including, intravenous drug abusers (n=30), North American Indians (n=13), Guaymi Indians from Panama (n=22) and routine U.S. blood donors (n=34) when compared with Env-20$^{85-102}$ (73% to 100%) or Env-203$^{219-256}$ (68% to 83%). Furthermore, Env-20$^{85-102}$ and Env-202$^{173-209}$ had minimal reactivity with sera from HTLV-I-infected individuals, whereas Env-203$^{219-256}$ reacted with 58% of HTLV-I specimens. We conclude that peptides Env-20$^{85-102}$ and Env-202$^{173-209}$ represent the type-specific immunodominant epitopes of HTLV-II external glycoprotein.

EXAMPLE 9

Material and Methods

Human Serum Specimens

A total of 145 serum specimens, including 123 HTLV-I/II seropositive and 22 routine blood donors, were chosen for this study (Table 3).

Table 3

Characteristics of serum specimens studied

Group	Number Tested	HTLV Ab* (gag & env)	PCR Analysis HTLV-I	PCR Analysis HTLV-II	Geographic area
Blood Donors	58	+	24	34	Mixed
IV Drug Users	30	+	0	30	U.S.
American Indians					
Guaymi	22	+	0	22	Panama
Seminole	4	+	0	4	Florida
Navajo	4	+	0	4	New Mexico
Pueblo	5	+	0	5	New Mexico
Normal Donors	22	-	ND	ND	U.S.

*Seropositivity defined by antibody to $p24^{gag}$ and $gp46^{env}$ &/or $gp68^{env}$.

The 123 seropositive individuals had diverse geographic origins and risk factors, and included 58 blood donors, 30 IV drug users, and 35 American Indians (22 Guaymi Indians from Panama, 4 Seminole Indians from Florida, and 4 Navajo and 5 Pueblo Indians from New Mexico). None of these specimens were from a person infected with both HTLV-I and HTLV-II.

Polymerase Chain Reaction

All seropositive specimens were confirmed to be from HTLV-I- or HTLV-II-positive persons by polymerase chain reaction (PCR) assays performed with DNA derived from peripheral blood lymphocytes from these individuals. Two gene regions from each patient were amplified using pol and tax/rex primers, and hybridized with ^{32}P end labeled oligoprobes from respective regions. The hybridized products were electrophoresed and autoradiographed as described previously (Kwok et al., J. Infect. Dis., 158, 1193-1197, 1989; Lee et al., Science, 244:471-475, 1989). Specimens were classified as HTLV-I or HTLV-II based on type-specific amplification of both pol and tax/rex regions.

Reference HTLV Antibody Tests

Serum specimens from all patients were initially tested for HTLV-I antibodies with a commercial enzyme-linked immunosorbent assay (HTLV-I ELISA, Dupont, Wilmington, DE), according to the manufacturer's recommendations. Specimens that were repeatedly reactive were further tested by immunoblotting and radioimmunoprecipitation assay as described previously (Lal et al., J. Infect. Dis., 163:41-46, 1991). A serum specimen was determined to be HTLV-I/II-positive if antibody reactivity was detected to gag p24 and env gp46 and/or gp68.

Peptide Selection and Synthesis

Several peptides were selected for synthesis based on antigenic index characteristics or by identifying regions in which HTLV-I and HTLV-II showed amino acid sequence differences (Lal and Griffis, **in press** 1991). Synthetic peptides were made on the MillGen 9050

Pepsynthesizer with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry according to recommended chemistry.

Quantitative Assessment of Antibodies to Synthetic Peptides

An enzyme immunoassay (EIA) was developed to detect antibodies to synthetic peptides as described previously (Lal et al., J. Infect. Dis., 163, 41-46, 1991). Briefly, polyvinyl plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, VA.) were coated with 50 m ml of synthetic peptides (5 mg/ml for Env-202, Env-203, Env-204, Env-208, and Env-212; all other peptides were coated with 100 mg/ml) in 0.01M carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) six times, and each well was incubated with 200 ul of 3% bovine serum albumin in PBS-T for 1 hr at 37°C to block excess reactive sites. After the wells were washed, a 1:20 dilution of each test serum was added to duplicate wells and the plates were incubated overnight at 4°C, followed by addition of p-nitrophenyl phosphate (Sigma) substrate. The plates were read with an ELISA reader (SLT Lab Instrument, Austria) at 405 nm. Each serum specimen was also assayed in plates coated with BSA alone or an unrelated synthetic peptide to control for nonspecific antibody binding. Seropositivity was defined as any value greater than the mean of the normal controls +2 standard deviations.

RESULTS

A noncompetitive EIA was developed with immobilized synthetic peptides to detect antibodies in persons infected with HTLV-II (n=99) or HTLV-I (n=24) and in healthy blood donors (n=22). Initial analysis of synthetic peptides derived from the HTLV-II^{gp52} with 30 serum specimens from HTLV-II-infected IVDA individuals identified three major domains of strong reactivity (Table 4).

Table 4

Seroreactivity of synthetic peptides derived from the envelope glycoprotein of HTLV-II

Peptide	a.a	Sequence	Seroreactivity* n=30 (% reactivity)
gp46^{env}			
Env 4	3-19	NVFFLLLFSLTHFPLAQ	2 (7)
Env 7	45-60	TWNLDLNSLTTDQRLH	3 (10)
Env 20	85-102	KKPNRQGLGYYSPSYNDP	22 (73)
Env 21	120-135	YTGPVSSPSWKFHSDV	5 (17)
Env 202	173-209	TSEPTQPPPTSPPLVHDSDLEHVLTPSTSWTTKILKF	29 (97)
Env 2	187-209	VHDSDLEHVLTPSTSWTTKILKF	28 (93)
Env 203	219-256	YSCMVCVDRSSLSSWHVLYTPNISIPQQTSSRTILFPS	18 (60)
Env 204	232-255	SWHVLYTPNISIPQQTSSRTILFP	3 (10)
Env 22	261-277	APPSQPFPWTHCYQPRL	2 (7)
Env 23	274-289	QPRLQAITTDNCNNSI	3 (10)
gp21^{env}			
Env 24	296-312	LAPVPPPATRRRRAVPI	1 (3)
Env 208	361-369	HQNILRVAQ	3 (10)
Env 25	369-384	AQYAAQNRRGLDLLFW	3 (10)
Env 212	397-408	CFLNISNTHVSV	2 (7)

*Seropositivity was defined as any value greater than the mean of the normal control + 2 standard deviation.

An epitope located at the N-terminus of gp52, as represented by peptide Env-20$^{85-102}$, reacted with 73% (22 of 30) of specimens derived from HTLV-II infected individuals. A second epitope located in the central region of gp52, as represented by peptide Env-202$^{173-209}$, reacted with 97% (29 of 30) of HTLV-II specimens. Deletion of 13 amino acids from the N-terminus of Env-202 (Env-2$^{187-209}$) resulted in some loss of reactivity (93%; 28 of 30 HTLV-II sera). The third epitope defined by peptide Env-203$^{219-256}$, located at the C-terminus of gp-52 reacted with 60% (18/30) of sera from HTLV-II-infected individuals. The predominant reactivity of this epitope was located at the N-terminus because a smaller peptide Env-204$^{232-255}$ had minimal reactivity with HTLV-II specimens. All other peptides derived from the HTLV-II^{gp52} or HTLV-II^{gp21} had minimal reactivity with serum specimens from HTLV-II-infected individuals (Table 4).

To further investigate the heterogeneity of biological responses to the immunodominant epitopes (Env-20$^{85-102}$, Env-202$^{173-209}$, Env-203$^{219-256}$), serum specimens from individuals of diverse geographic origins and risk factors were evaluated (Table 5).

Table 5

Recognition of Synthetic HTLV-II Envelope Peptides by Antibodies in HTLV Seropositive Sera.

Group	Seroreactivity to peptide		
	Env-20 (%)	Env-202 (%)	Env-203 (%)
HTLV-II infected			
Blood Donors (n=34)	27 (79)	31 (91)	23 (68)
IVDU (n=30)	22 (73)	29 (97)	25 (83)
American Indians			
Panama (n=22)	22 (100)	22 (100)	18 (82)
U.S. (n=13*)	11 (85)	12 (92)	9 (69)
Total (n=99)	82 (83)	94 (95)	75 (76)
HTLV-I infected			
Blood Donors (n=24)	2 (8)	6 (25)	14 (58)
Normal Donors (n=22)	0	0	0

*Four of the specimens were from Seminole Indians in Florida, 4 from Navajo and 5 from Pueblo Indians in New Mexico.

The Env-202$^{173-209}$ reacted with most of the specimens from HTLV-II-infected individuals: 91% of blood donors, 97% of IV drug users, 100% of Guaymi Indians, and 92% of North American Indians. Env-2$^{187-209}$ also had similarly high rates of seroreactivity (data not shown). Env-20$^{85-102}$ reacted with 79% of blood donors, 73% of IV drug users, 100% of Guaymi Indians, and 85% of North American Indians; Env-203$^{219-256}$ reacted with 68% of blood donors, 83% of IV drug users, 82% of Guaymi Indians and 69% of American Indians. No specific differences in the antibody profiles to Env-20, Env-202 and Env-203 were discernable among any of study groups (data not shown).

Because of the high seroreactivity of HTLV-II specimens (62%-100%) with these peptides, we next assessed whether they could represent type specific epitopes. Structural analysis of primary amino acids of Env-20, Env-202, and Env-203 with corresponding regions of HTLV-I

demonstrated variable degrees of homology (78%, 43%, and 57%, respectively) (Figure 6). Further analysis of seroreactivity with HTLV-I specimens (Figure 7) demonstrated that Env-20 and Env-202 had minimal reactivity with low optical densities (8% and 25%, respectively), and Env-203 had significant reactivity with high optical densities (58%). None of the 22 healthy blood donors reacted with any of the peptides.

DISCUSSION

The emergence of HTLV-II as an equally prevalent virus as HTLV-I in U.S. blood donors (Sandler et al., Yale J. Biol. Med., 63:353-360, 1990) has made it necessary to not only devise better diagnostic tests, but also define immunodominant epitopes that may be important for understanding the host-virus interaction. In the present investigation we identified three epitopes that are recognized by serum antibodies in most HTLV-II infected individuals.

Env-20^{85-102}, located at the N-terminus of HTLV-II, reacted with 83% of specimens from HTLV-II-infected individuals. Surprisingly, a minimal reaction was observed with HTLV-I specimens despite significant homology of Env-20 with HTLV-I (Fig. 6). Although fine mapping of Env-20 was not carried out, it is expected that the antibody-combining site probably consists of amino acids that are not conserved between Env-20 and the homologous region of HTLV-I. A peptide (a.a. 89-110) from the homologous region of HTLV-I envelope contains an epitope that reacts with HTLV-I-positive sera (Horal et al., Human Retrovirology:HTLV (W.A. Blattner Ed.) p. 461-467, Plenum Press, NY, 1990) and polyclonal antibodies raised to another peptide (SP2, a.a. 86-107) from this region neutralized HTLV-I in both a syncytium inhibition assay and HTLV-I pseudotype assay (Palker et al., Presented at "Current Issues In Human Retrovirology: HTLV, Montego Bay, Jamaica, February 10-14, 1991).

Env-$202^{173-209}$, which reacted with 95% of HTLV-II infected specimens and had minimal reaction with HTLV-I

specimens, was the most dominant and type-specific epitope of the HTLV-II external glycoprotein. The immunodominance of the central region of the HTLV-II envelope has previously been documented (Lal et al., J. Infect. Dis., 163, 41-46, 1991; Chen et al., J. Virol., 63, 4952-4957, 1990). A recombinant protein RP-IIB containing amino acids 96-235 reacted with all HTLV-II sera, but also reacted with 65% of HTLV-I sera. We previously demonstrated that a peptide, Env-2 (a.a. 187-209), reacts with the majority of HTLV-II sera, with some cross-section with HTLV-I specimens (Lal et al., J. Infect. Dis., 163, 41-46, 1991). The additional 13 amino acids at the N-terminus of Env-202$^{173-209}$ markedly increased the specificity of its reactivity as compared with Env-2. The larger peptide presumably changes the conformation of the epitopes such that it no longer recognizes HTLV-I specimens. Because of this high seroreactivity and type specificity, Env-202 has recently been used successfully to develop an assay that can serologically discriminate between HTLV-I and HTLV-II (Viscidi et al., J. Acquir. Immune. Defic. Syndr. (in press). The central region of HTLV-I glycoprotein also contains several dominant epitopes, as defined by either recombinant proteins such as MTA-4 (Lipka et al., J. Infect. Dis., 162, 353-357, 1990) and RP-B1$^{166-201}$ (Chen et al., Lancet, 336, 1153-1155, 1990) or synthetic peptides such as SP4a$^{190-209}$ (Palker et al., J. Immunol., 142:971-978, 1989) and Env-1$^{191-215}$ (Lal et al., J. Infect. Dis., 163, 41-46, 1991) have all demonstrated predominant seroreactivity with HTLV-I-infected individuals. In addition, the epitope defined by SP4a$^{190-209}$ has further been shown to contain a neutralizing epitope (Ralston et al., J. Biol. Chem., 264, 16343-16346, 1989), human cytotoxic T cell epitope (Jacobson et al., J. Immunol., 146, 1155-1162, 1991), and murine T helper epitope (Kurata et al., J. Immunol., 143, 2024-2030, 1989).

The third motif represented by Env-203$^{219-256}$ reacted almost equally with HTLV-II and HTLV-I specimens. Since a smaller peptide (Env-204) with a.a. 232-255 had minimal

reactivity with HTLV-infected specimens, the antibody-binding domain of Env-203 appears to be located toward the N-terminus. A type-specific immunodominant epitope of HTLV-I external glycoprotein was mapped to the C-termini of the envelope protein (Env-$5^{242-257}$) and has subsequently been shown to be exposed on the cell surface of HTLV-I infected cells (Lal et al., J. Infect. Dis., 163, 41-46, 1991; J. Gen. Virol., (in press)).

None of the four synthetic peptides derived from the transmembrane protein of HTLV-II demonstrated significant binding with serum specimens from HTLV-II infected persons. While little is known about the immunogenicity of the HTLV-II transmembrane protein, recombinant RP-$D^{313-488}$ (Chen et al., J. Virol., 63, 4952-4957, 1989), $rgp21^{307-440}$ (Samuel et al., Science, 226, 1094-1097, 1984), and synthetic $TM101^{379-403}$ (Viscidi et al., J. Acquir. Immune. Defic. Syndr., (in press)) have all shown reactivity with serum specimens from both HTLV-I- and HTLV-II-infected individuals. Our inability to detect antibodies to synthetic peptides from the analogous region of the HTLV-II transmembrane protein may be due to the small size of the peptides used. Alternatively, the conformation of the peptide once it is bound to the solid phase may be such that it no longer reacts with the antibodies in the sera. Env-$25^{369-384}$ represents the putative immunosuppressive domain that inhibits both lymphoproliferation (Ruegg et al., J. Virol., 63, 3250-3256, 1989) and immunoglobulin secretion (Mitani et al., Proc. Natl. Acad. Sci. USA, 84, 237-240, 1987) and the active site for inhibition of lymphoproliferation has been mapped to 10 amino acids (AQNRRGLDLL) (Ruegg et al., J. Virol., 63, 3250-3256, 1989).

One practical application of these findings regarding the immunodominant regions of the HTLV-II external glycoproteins may be development of type-specific assays. Thus, two of these motifs defined by Env-20^{85-102} and Env-$202^{173-209}$ represent HTLV type II-specific epitopes and could be used in combination for developing serologic

assays to distinguish infection with HTLV-II from that with HTLV-I.

In order to better understand the clinical importance of HTLV^{ind}, a group of low-risk blood donors within U.S. armed forces with isolated HTLV^{gag} reactivity was evaluated by a number of serologic assays containing whole viral antigens and the immunodominant epitopes of HTLV-I and HTLV-II. The presence of viral DNA in the genome was analyzed by the polymerase chain reaction, which allows rapid and direct detection of viral DNA through the amplification of specific viral sequences in blood samples (Lee et al., Science, 244:471-475, 1989; Kwok et al., J. Infect. Dis., 158:1193-7, 1988). In Example 10, analysis of blood donors with HTLV^{ind} pattern demonstrated no evidence of HTLV-I or HTLV-II infection according to HTLV antibody enzyme immunoassay with synthetic antigens representing the immunodominant epitopes of HTLV-I and HTLV-II, and by PCR assays.

Of the 267,650 blood donations from members of the U.S. armed forces, 27 (0.027%) were serologically confirmed to be positive for human T-lymphotropic virus type-I/II (HTLV^{pos}) and 379 (0.027%) were western blot (WB) indeterminant with banding pattern restricted to the proteins encoded by the gag gene only (HTLV^{ind}). To determine whether these apparently healthy HTLV^{ind} blood donors are infected with HTLV-I or HTLV-II, coded specimens from 73 such army blood donors were tested for antibody to HTLV by western blot and radioimmunoprecipitation assay using HTLV-I (MT-2) and HTLV-II (Mo-T) antigens, by enzyme immunoassay using synthetic peptides representing the immunodominant epitopes of HTLV, and for sequences of HTLV DNA by the polymerase chain reaction.

Of the 73 HTLV^{ind} donors, none showed presence of env reactivity by HTLV-I or HTLV-II WB and RIPA. Minimal reactivity was observed with synthetic immunodominant motifs derived from the env protein (Env-1$^{191-213}$, Env-5$^{242-257}$, Env-2$^{187-209}$, and Env-20$^{85-102}$) and gag protein (Gag-1a$^{102-117}$, and

Gag-10$^{364-385}$). An endogenous retroviral sequence with structural homologies to the gag protein of HTLVs (RTVL^{gag}) demonstrated antibodies not only in HTLV^{ind} specimens, but also reacted with normal control subjects. Furthermore, none of the 73 HTLV^{ind} specimens demonstrated presence of HTLV genome when amplified in the pol and tax/rex region. After 6 to 23 months from the time of the initial test, 23 subjects still gave similar WB patterns, and 9 of these repeat specimens were still negative for the presence of HTLV genome. Thus, persons at low risk for HTLV infection who have HTLV^{ind} western blot reactivity are rarely, if ever, infected with HTLV-I and HTLV-II.

The criteria for seropositivity, as defined by the U.S. Public Health Service Working group, is that a serum specimen exhibiting reactivity to p24^{gag} and gp46^{env} and/or gp61/68^{env} can be considered seropositive for HTLV-I/II and that a combination of Western blotting (WB) and radioimmunoprecipitation assays (RIPA) be used to visualize antibody reactivity to gag and env (Anderson et al., Blood, 74:2585-91, 1989; CDC, MMWR, 39:915, 921-924, 1990); any pattern which lacks this full array of band by either test system are considered western blot indeterminant (HTLV^{ind}). Such testing of blood donors within the U.S. has revealed a low seroprevalence of 0.01% to 0.025% western blot-positive donations (Anderson et al., Blood, 74:2585-91, 1989; Sandler et al., 63:353-60, 1990). However, greater than 50% of initial EIA reactive specimens demonstrate reactivity to one or two band characteristics of HTLV-I^{gag} on WB assays (Sandler et al., 63:353-60, 1990; Hartley et al., 28:646-50, 1990). While the importance of such reactivity remains to be determined, retrospective studies of recipients of blood with such serum reactivity showed that these recipients were HTLV-I/II negative (Shih et al., Blood, 75:546-9, 1990).

EXAMPLE 10

Methods

Reference HTLV Antibody Tests

Serum specimens from all blood donors were initially tested for HTLV-I/II antibodies with a licensed enzyme linked immunosorbent assay (HTLV-I ELISA, Dupont, Wilmington, DE), according to the manufacturer's recommendations. Specimens that were repeatedly reactive were further tested by WB incorporating purified recombinant HTLV-I envelope (r21) protein with a whole virus lysate derived from an HTLV-I infected cell line, HuT-102 (Cambridge Biotech, Rockville, MD) and RIPA using a lysate from the MT-2 cell line (Cambridge). A serum specimen was determined to be HTLV-positive if antibody reactivity was detected to at least two different HTLV structural gene products (gag p24 and env gp46 and/or gp68). A donor's assay results were considered to be indeterminant ($HTLV^{ind}$) for HTLV-I/II, if the WB showed at least one band characteristic of HTLV-I/II (p19, p24, or gp46) but did not meet the criteria for a positive result. Specimens wiht $HTLV^{ind}$ patterns were further analyzed on WB and RIPA containing HTLV-II antigens derived from an Mo-T cell line.

Blood Donors

Between December, 1988 and April, 1991, approximately 267,650 units of blood from members of the armed forces were tested for antibody to HTLV-I/II at the Walter Reed Army Medical Center, Washington. Of the 267,650 donations, 2376 (0.89%) were initially reactive on enzyme immunoassay and hence were tested by the western blot and RIPA for serologic confirmation of HTLV infection (Table 6).

Table 6

HTLV-I/II immunoassay and PCR results in armed forces blood donors

Variables	WB/RIPA Results Original	WB/RIPA Results 3-23 mos. later	PCR Results Original	PCR Results 3-23 mos. later
$HTLV^{pos}$	72 (0.027)			
HTLV-I	43	30	36/36	28/28
HTLV-II	29	25	16/16	13/13
$HTLV^{ind}$	379 (0.14)	23	0/73	0/9
p21+p19+p24+	4	ND	0/3	ND
p21+p19+p24+	10	ND	0/1	ND
p21+p19+p24+	7[2]	ND	0/4	ND
p19+p24+	28	2	0/10	0/1
p24+	61	5	0/12	0/2
p21+	12	ND	0/5	ND
p19+	257[1]	16	0/38	0/6
$HTLV^{neg}$	1925	ND	0/26	ND

[1] 128 specimens within this group had some nonspecific bands at 28kD and 26kD, in addition to p19 bands.

[2] 2 specimens were from persons infected with HIV.

Of these, 72 were HTLV^{pos} (0.027%), 379 were HTLV^{ind}, and 1925 did not demonstrate any viral specific band (HTLV^{neg}).

Antibodies to Synthetic Peptides

The synthetic peptides derived from HTLV-I and HTLV-II sequences termed Env-1$^{191-213}$ (HTLV-I, LPHSNLDHILEPSIPWKSKLLTLV), Env-2$^{187-209}$ (HTLV-II, VHDSDLEHVLTPSTSWTTKILKF), Env-5$^{242-257}$ (HTLV-I, SPNVSVPSSSSTPLLY), Env-20$^{85-102}$ (HTLV-II, KKPNRQGLGYYSPSYNDP), Gag-1a$^{102-117}$ (HTLV-I, PPSSPTHDPPDSDPQI), Gag-10$^{364-385}$ (HTLV-I/II, GHWSRDCTQPRPPPGPCPLCQDP) and an endogenous retroviral sequence containing histidine tRNA primer binding site with sequence homologies to C-terminus of HTLV^{gag} protein (RTVL^{gag}, PRIPPKPCPICVGPNWKSDCPT) were synthesized by FMOC chemistry and antibodies to these synthetic peptides were tested as described previously (Lal et al., J. Virol., 65:1870-1876, 1991). Briefly, polyvinyl plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 50 ml of synthetic peptides (100 ug/ml) in 0.01M carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 (PBS-T) six times, excess reactive sites were blocked by addition of 3% BSA in PBS-T, followed by addition of a 1:20 dilution of each test serum and the plates were incubated overnight at 4°C. After six washes, Fc specific, alkaline phosphatase conjugated goat antibody to human IgG (Sigma, St. Louis) was added, followed by addition of p-nitrophenyl phosphate (Sigma) substrate. The plates were read with an ELISA reader (SLT Lab Instrument, Austria) at 405 nm. Seropositivity was defined as any value greater than the mean of the normal controls +2 standard deviations.

Polymerase chain reaction

The amplification and detection of HTLV sequences by the PCR were performed in blinded fashion on DNA specimens from HTLV^{pos} donors (n=52), and HTLV^{ind} donors (n=73). Two gene regions (pol and tax-rex) from each patient were amplified by PCR using conditions as

described previously (Lee et al., Science, 244:471-475, 1989; Kwok et al., J. Infect. Dis., 158:1193-7, 1988). Briefly, fifty ml of cell lysate were added to 50 ml of the reagent mixture containing dNTP's, primers, and Taq polymerase (all 2X) in 1X PCR buffer (50 nM KC1, 10 mM Tris-HCl, pH 8.3, 0.1 mg/ml gelatin). The optimal concentrations determined were: dNTP's, 200 mM each; primers, 0.5 uM each; Taq polymerase, 1U; and $MgCl_2$, 1.25 mM. The amplification conditions followed were: denaturatoin of 94°C for 90 sec.; annealing at 58°C for 2 min; extension at 72°C for 1 min, for 40 cycles. Ten microliters of PCR products were hybridized with ^{32}P end-labeled oligonucleotide probes in solution at 53°C and 45°C respectively. The 5'-3' sequences of the primer pairs and probes, based on HTLV-I (GenBank accession no. J02029) and HTLV-II (GenBank accession no. M10060) sequences were as follows: SK110 (pol, HTLV-$I^{4757-4778}$, HTLV-$II^{4735-4756}$)-CCCTACAATCCAACCAGCTCAG; SK111 (pol, HTLV-$I^{4942-4919}$, HTLV-$II^{4920-4897}$)-GTGGTGAAGCTGCCATCGGGTTTT; SK112 (pol, HTLV-$I^{4825-4840}$)-GTACTTTACTGACAAACCCGACCTAC; SK188 (pol, HTLV-$II^{4880-4998}$)-TCATGAACCCCAGTGGTAA. The hybridized products were electrophoresed on 10% polyacrylamide gels and autoradiographed.

The second amplification was performed in the tax/rex region as described previously. Briefly, fifty ml of the cell lysate was added to the $2x10^6$ cpm of 5' labelled primer, dNTPs (50mM), Taq polymerase (2.5 U) and $MgCl_2$ (1.25mM). The primers used were Tx1 (tax/rex, HTLV-$1^{7336-7353}$, HTLV-$II^{7248-7266}$)-CGGATACCCAGTCTACGT; and Tx2 (tax/rex, HTLV-$1^{7494-7474}$, HTLV-$II^{7406-7386}$)- GAGCCGATAACGCGTCCATCG. The amplification conditions were similar to as described above, except the annealing temperature was 55°C. The amplified products were digested with restriction enzymes Taq I and Sau 3A, and the products were electrophoresed on 8% polyacrylamide gels and autoradiographed. A specimen was considered positive by PCR if HTLV sequences were detected by both primer parts. If a specimen was positive

on one of the two amplifications, a third amplification was performed to determine positivity or negativity. A specimen was considered to be HTLV-negative by PCR if there were no detectable HTLV sequences when cells were analyzed in duplicate for each of two primer pairs.

RESULTS

Characteristic western blot indeterminate pattern

The most common indeterminant band pattern showed a single band at p19 (257/379, 68%), followed by a single band at p24 (61/379, 16%), bands at p19 and p24 (28/379, 7%), bands at p19 and/or p24 along with reactivity to rgp21 (21/379, 5.6%) and a single band at rgp21 (12/379, 3%). None of the specimens with $HTLV^{ind}$ pattern demonstrated any envelope reactivity on HTLV-II WB or RIPA analysis using both HTLV-I and HTLV-II lysates.

Western blot analysis was repeated in 23 of the 379 $HTLV^{ind}$ specimens drawn 6 to 23 months after the original bleed. No differences in the banding pattern were seen in 21 of 23 labeled specimens, one of the specimens with p19+p24+ pattern lost p24 reactivity and one of the p19+ specimens acquired r21+ reactivity upon rebleed.

Antibody response to immunodominant HTLV epitopes

As affinity and avidity of antibodies in serum may affect its detection in standard serologic assays using whole viral lysates, antibody responses were determined to synthetic immunodominant structural motifs of HTLV-I and HTLV-II. While 92% to 99% of HTLV-I infected persons reacted with HTLV-I specific env epitopes (Env-$1^{191-213}$, and Env-$5^{242-256}$), minimal reactivity (0 to 12%) was observed with specimens with $HTLV^{ind}$ pattern (Fig. 8). Similarly, while 75% to 96% of HTLV-II infected persons reacted with HTLV-II specific env epitopes (Env-$2^{187-209}$, and Env-20^{85-102}), minimal reactivity (0 to 5%) was observed with $HTLV^{ind}$ specimens. Analysis of synthetic peptides derived from the C-terminus of p19 (Gag-$1a^{102-117}$) and the N-terminus of p15 (Gag-$10^{364-385}$) demonstrated 65 to 95% reactivity with $HTLV^{pos}$ specimens. Among the $HTLV^{ind}$ specimens, 23% of those

with p19 band only reacted with Gag-1a, all other specimens had minimal reactivity with Gag-1a or Gag-10.

The expression of endogenous retroviral gene products may also provide an antigenic stimulus for production of antibodies that may be cross reactive with $HTLV^{neg}$ proteins. Peptides derived from an endogenous retroviral element were synthesized having a histidine tRNA primer binding site (RTVL-H) that has 60% homology with the C-terminus of HTLV-I and HTLV-II ($RTVL^{neg}$). While 88% of $HTLV^{pos}$ specimens reacted with this peptide, 42% to 66% serum specimens from $HTLV^{ind}$ specimens also reacted with $RTVL^{neg}$. However, further analysis of $HTLV^{neg}$ specimens demonstrated that 60% of these specimens also react with this peptide.

Detection of HTLV DNA Sequences in Blood Donors

To determine the presence or absence of HTLV DNA in persons with $HTLV^{ind}$ pattern on WB, peripheral blood lymphocytes were analyzed by PCR. Primer pairs were chosen from the pol and tax-rex region, both of which are highly conserved among HTLV-I and HTLV-II. Regions from gag and env were not amplified due to some sequence homology with endogenous retroviral sequences and variation in different isolate sequences, respectively. In accordance with previous studies, both primers from the pol and tax/rex region were highly sensitive in identifying $HTLV^{pos}$ specimens (all of the 52 $HTLV^{pos}$ gave a detectable signal). None of the 15 $HTLV^{pos}$ gave a detectable signal). None of the 15 $HTLV^{neg}$ specimen reacted with any of the primer/probe combinations, further confirming the specificity of these primers. Of the 73 $HTLV^{ind}$ specimens with different band patterns (Table 6), none amplified any product with either primer/probe combination. PCR analysis of the repeat specimen drawn 6 to 23 months after the original testing on 9 of the $HTLV^{ind}$ specimens did not demonstrate presence of HTLV genome in any of the specimens.

DISCUSSION

The serologic confirmation of HTLV-I and HTLV-II infection depends on the presence of antibody reactivity to gag and env gene products (Anderson et al., Blood, 74:2585-91, 1989; CDC, MMWR, 39:915, 921-924, 1990). Using these criteria, 72 of the total donations (0.027%) were HTLV positive, giving an overall seroprevalence rate of 0.027%. Further analysis of these seropositive specimens by type specific oligopeptide and oligoprimers demonstrated that 43 were infected with HTLV-I (60%), whereas 29 were HTLV-II (40%). These rates are in gneral agreement with previous studies, where random U.S. blood donors were shown to have seropositivity rates of 0.01 to 0.02% with an equal distribution of HTLV-I and HTLV-II (Anderson et al., Blood, 74:2585-91, 1989; Sandler et al., J. Biol. Med., 63:353-60, 1990).

In addition, persons with isolated gag reactivity are frequently encountered during blood donor screening assays and are referred to as HTLV indeterminant ($HTLV^{ind}$). A significant number of blood donors were $HTLV^{ind}$ in the present study. The most common reactivity is directed against the $p19^{gag}$, followed by antibody reactivity to $p24^{gag}$. The structural similarity of epitope(s) with other microbial and cellular proteins (Lal et al., J. Virol., 65:1870-76, 1991; McLauglin et al., Amer. J. Trop. Med. Hyg., 37:258-62, 1987) and the immunogenic nature of the C-terminus of $p19^{gag}$ may account for this reactivity (Lal et al., J. Med. Virol. in press). Indeed, 23% of the specimens with p19 reactivity in WB assay demonstrated antibody responses to a synthetic $Gag\text{-}1a^{102\text{-}117}$ epitope, that has previously been shown to represent a type specific immunodominant epitope of HTLV-I (Lal et al., J. Virol., 65:1870-76, 1991). Furthermore, monoclonal antibodies to $p19^{gag}$ have been shown to react with antigens of normal thymus or human placenta (Haynes et al., J. Exp. Med., 157:907-20, 1983; Suni et al., Int. J. Cancer, 33:293-8, 1984).

In addition, the C-terminus of $HTLV^{gag}$ representing

the quasi-periodic primary structure has significant homologies with the amino-terminal segment of myelin basic protein (MBP) and may have potential for false positive antibodies (Liquori, J. Theor. Biol., 148:279-81, 1991).

Isolated gag reactivity in the absence of env reactivity could either be due to inability of current assays available for detection of env reactivity, or due to cross reactivity with closely related retrovirus or may represent early HTLV infection. Lack of antibody response to the envelope proteins in WB and RIPA assays using both HTLV-I and HTLV-II antigens, as well as minimal reaction to synthetic immunodominant motifs derived from the envelope protein of HTLV-I and HTLV-II (Env-1, Env-2, Env-5, Env-20) further confirm that persons with isolated gag antibodies do not contain true HTLV env reactivity. However, the possibility that the lack of env reactivity might be due to minimal viral load resulting in lack of threshold of an immunogenic signal cannot be ruled out. Alternatively, persons with isolated gag reactivity might be infected with variant form of HTLV viruses that have significant divergence in the envelope protein and therefore do not react with prototypic viral strains. Such variant forms of viruses have lately been isolated from Papua, New Guinea (Yanagihara et al., Proc. Natl. Acad. Sci. USA, 88:1446-50, 1991).

Since gag antibodies are among the first antibodies to appear following sero-conversion (Manns et al., Blood, 77:896-905, 1991), the possibility that isolated core antibodies in $HTLV^{ind}$ specimens may represent early sero-converters remains a possibility. However, PBMC from none of the specimens with isolated gag reactivity demonstrated presence of HTLV genome as determined by PCR analysis. More importantly, rebleeds on a limited number of specimens after 6 to 23 months of initial testing have demonstrated similar banding patterns on WB, do not show env reactivity on RIPA, and remain negative by PCR analysis. A retrospective study of recipients of blood donors with $HTLV^{ind}$ patterns have demonstrated no evidence of

seroconversion to HTLV when followed up for a year (Sandler et al., J. Biol. Med., 63:353-60, 1990). Antibodies to a recombinant transmembrane glycoprotein (r21) have recently been shown to be another marker of early seroconversion to HTLV (Manns et al., Blood, 77:896-905, 1991), though the specificity of r21 detection is yet to be confirmed. In the present study, none of the specimens with $r21^{env}$ and gag antibody demonstrated presence of HTLV genome by PCR analysis. None of the specimens with $r21^{env}$ reactivity could be followed up, therefore, it cannot be concluded whether such reactivity represents true seroconversion.

The antigenic mimicry of endogenous retroviral sequences with gag proteins of HTLV might be responsible for gag reactive antibodies (Abraham et al., Clin. Immunol. Immunopathol., 56:1-8, 1990). Antibody response was found to a synthetic peptide derived from the endogenous retroviral sequence ($RTVL^{gag}$, Mager et al., J. Virol., 61:4060-4066, 1987) both in $HTLV^{pos}$ and $HTLV^{ind}$ specimens. The endogenous retroviral sequence has 60% homology with a 50 amino acid sequence at the C-terminus of gag. This gag region of HTLV's containing the highly conserved retroviral motif $CX_2CX_4HX_4C$, present in other type C retroviruses, as well as in human immunodeficiency virus, is though to be involved in binding of this protein to the retroviral genome (Covey, Nucleic Acids Res., 14:623-33, 1986). The RTVL region contains two imperfect copies of this conserved sequence in a location very similar to that found in other retroviruses (Fig. 9). In addition, another ERS contains two open reading frames potentially encoding for 25kD and 15kD and show 32-39% homology with the gag protei of HTLV-I/II (Perl et al., Nucleic Acids Res., 17:6841-54, 1989). While a majority of ERS are structurally defective and not expressed as infectious viruses, the expression of certain portions of the ERS gene products may provide an antigenic stimulus for production of antibodies. $RTVL^{gag}$ appears to be one of such epitopes that induce antibody response both in HTLV

infected and uninfected persons.

In the search for an HTLV genome in persons with $HTLV^{ind}$ pattern, the pol and tax/rex regions were focussed upon as these are highly conserved in HTLV-I and HTLV-II. While all of the $HTLV^{pos}$ specimens demonstrated presence of HTLV genome, none of the specimens with $HTLV^{ind}$ amplified with either pol or tax/rex primers. Attempts to isolate HTLV virus by a co-culture technique from a limited number of specimens did not show evidence of HTLV antigens in culture supernatants (unpublished). Because of the cell associated nature of HTLV, virus isolation is not a sensitive procedure for detecting HTLV. Though the mechanism of these serum reactivities to gag proteins remains unknown, the failure of the PCR to detect evidence of HTLV infection in $HTLV^{ind}$ specimens, as observed by the present inventor and by others (Kwok et al., Transfusion, 30:491-6, 1990), Khabbaz et al., submitted), seems to rule out the possibility that these reactivities correspond to an incomplete expression of HTLV gag proteins. While continued efforts are needed to determine the cause of these atypical HTLV WB results, the failure to detect env reactivity using whole viral antigens or synthetic immunodominant structural motifs of HTLV-I and HTLV-II, and the failure to amplify HTLV sequences from these individuals suggests that isolated gag reactivities do not represent true HTLV infection.

All publications, including U.S. Patents, as well as all U.S. Patent applications referred to in this application are herein incorporated by reference.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

WHAT IS CLAIM IS:

1. A peptide having specific immunoreactivity to antibodies to HTLV-I, HTLV-II, or combinations thereof comprising a peptide selected from the group consisting of:

Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV,
Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI,
Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY,
Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI,
Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK,
Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP,
Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI,
Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP,
Ers (endogenous retroviral sequence)
PRIPPKPCPICVCPNWKSDCPT, and

analogues thereof, wherein the amino acids in the sequence may be substituted as long as the immunoreactivity to antibodies to HTLV-I or HTLV-II derived from the three dimensional conformation of the sequences are substantially preserved.

2. The peptide of claim 1, which is Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV.

3. The peptide of claim 1, which is Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI.

4. The peptide of claim 1, which is Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY.

5. The peptide of claim 1, which is Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI.

6. The peptide of claim 1, which is Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK.

7. The peptide of claim 1, which is Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP.

8. The peptide of claim 1, which is Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI.

9. The peptide of claim 1, which is Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP.

10. The peptide of claim 1, which is Ers (endogenous retroviral sequence)PRIPPKPCPICVCPNWKSDCPT.

11. An immunoassay method for the detection of antibodies to HTLV-I, HTLV-II or combinations thereof which comprises:

(i) providing an effective amount of a peptide of claim 1 for reacting with antibodies to HTLV-I, HTLV-II or combinations thereof in an amount sufficient to produce an antibody-peptide complex to be detected,

(ii) adding a test sera diluted with a buffer wherein the antibodies to HTLV-I or HTLV-II in the test sera form a peptide-antibody complex with said peptide,

(iii) incubating the mixture, and

(iv) detecting the presence of the peptide-antibody complex.

12. The immunoassay according to claim 11, wherein said peptide is selected from the group consisting of Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT.

13. The immunoassay according to claim 11, wherein in step (iv), a second known antibody labelled with an enzyme and a substrate is introduced which reacts with the enzyme to form a colored product.

14. The immunoassay according to claim 11, wherein in step (iv), a second known antibody labelled with a radioactive element is introduced.

15. The immunoassay according to claim 11, wherein the peptide antibody complex is detectable as agglutination.

16. The immunoassay according to claim 11, wherein the solid support is a strip coated with at least one of said peptides in a multidot array.

17. The immunoassay according to claim 11, wherein the amount of said peptide coated on said solid support is in the range of 1 μg to 10 μg per well dot.

18. A test kit for the detection of antibodies to

HTLV-I, HTLV-II, or combinations thereof, which comprises:

an immunoadsorbent comprising at least one peptide of claim 1,

a sample of normal serum as a negative control;

a sample of serum containing antibodies to HTLV-I or HTLV-II as a positive control, and

a buffer for diluting the serum samples.

19. The test kit according to claim 18, wherein said peptide is selected from the group consisting of Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT.

20. A peptide composition comprising a mixture of at least two of the peptides of claim 1.

21. The peptide composition according to claim 20, wherein said peptide is selected from the group consisting of Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT.

22. The peptide composition of claim 20, wherein each of the peptides present in the mixture is present in a ratio of 1:1 with respect to one another and each peptide is present in an amount of 0.1 - 10 μg.

23. A vaccine comprising at least one of the

peptides of claim 1.

24. The vaccine according to claim 23, wherein said peptide is selected from the group consisting of Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT.

25. Use of at least one of the peptides of claim 1, for the preparation of a medicament for administration to a mammal for the treament of HTLV-I and HTLV-II infections.

26. Use of claim 25, wherein said peptide is selected from the group consisting of Env-1 (HTLV-I; a.a. 191-215)LPHSNLDHILEPSIPWKSKLLTLV, Env-2 (HTLV-II; a.a 187-210)VHDSDLEHVLTPSTSWTTKILKFI, Env5 (HTLV-I; a.a 242-257)SPNVSVPSSSSTPLLY, Gagla (HTLV-I; a.a 102-117)PPSSPTHDPPDSDPQI, Pol-3 (HTLV-I; a.a 487-502)KQILSQRSFPLPPPHK, Env-20 (HTLV-II; a.a. 85-102)KKPNRQGLGYYSPSYNDP, Env-23 (HTLV-I; a.a. 274-289)QPRLQAITTDNCNNSI, Gag-10 (HTLV-I/II; a.a. 364-385)GHWSRDCTQPRPPPGPCPLCQDP, Ers (endogenous retroviral sequence) PRIPPKPCPICVCPNWKSDCPT.

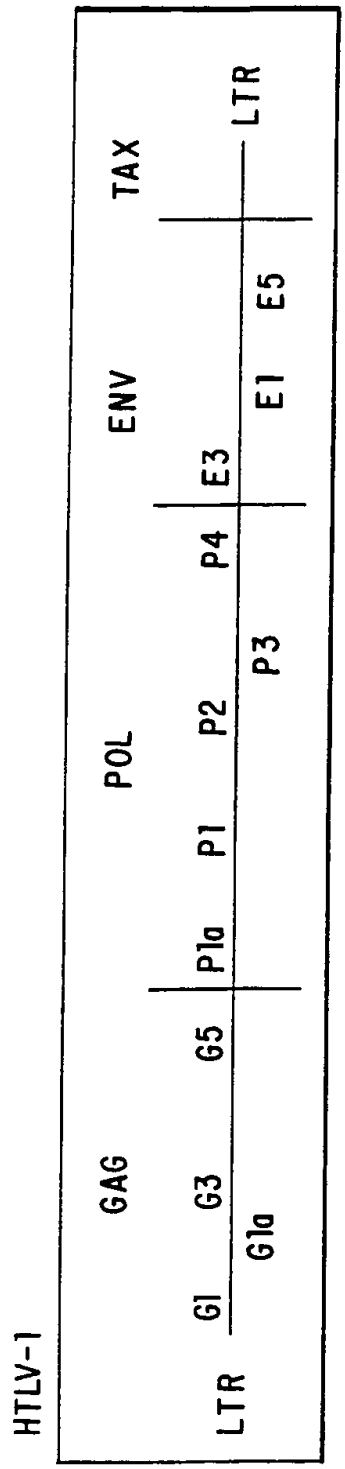
HTLV-1
LTR
G1
G1a
G3
G5
GAG
P1a
P1
P2
POL
P3
P4
E3
ENV
E1
E5
TAX
LTR

FIG. 1A

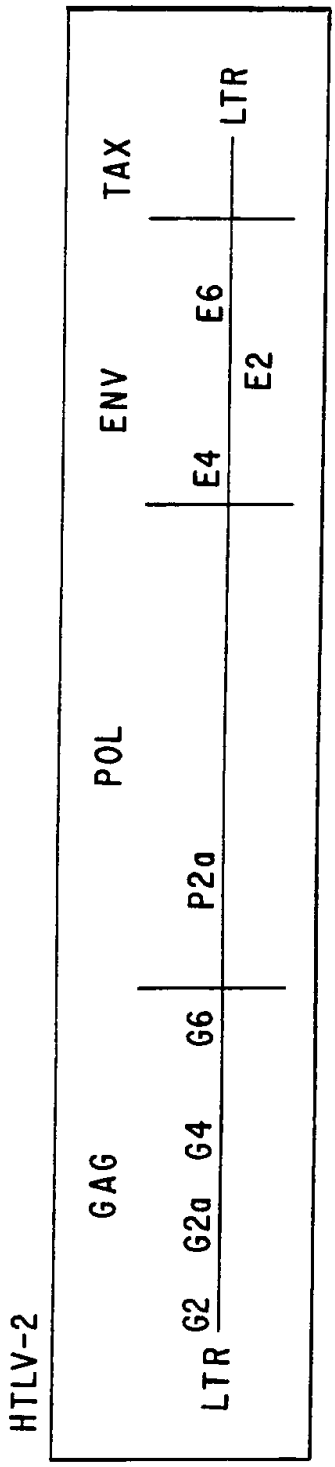
HTLV-2
LTR
G2
G2a
G4
G6
GAG
P2a
POL
E4
ENV
E2
E6
TAX
LTR

FIG. 1B

FIG. 2A

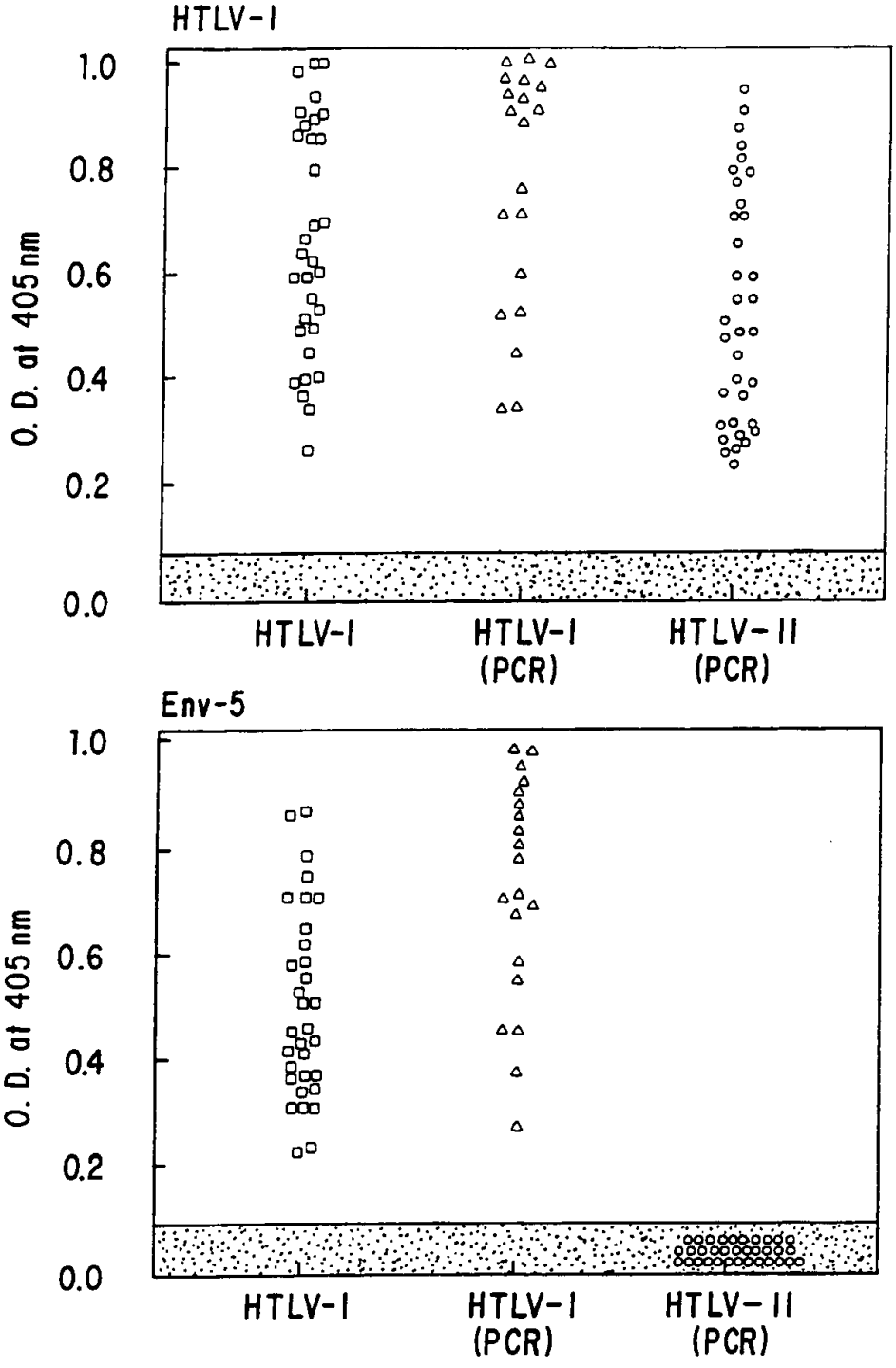
HTLV-I
1.0
0.8
0.6
0.4
0.2
0.0
O. D. at 405 nm
HTLV-I
HTLV-I (PCR)
HTLV-II (PCR)
Env-5
1.0
0.8
0.6
0.4
0.2
0.0
O. D. at 405 nm
HTLV-I
HTLV-I (PCR)
HTLV-II (PCR)

FIG. 2B

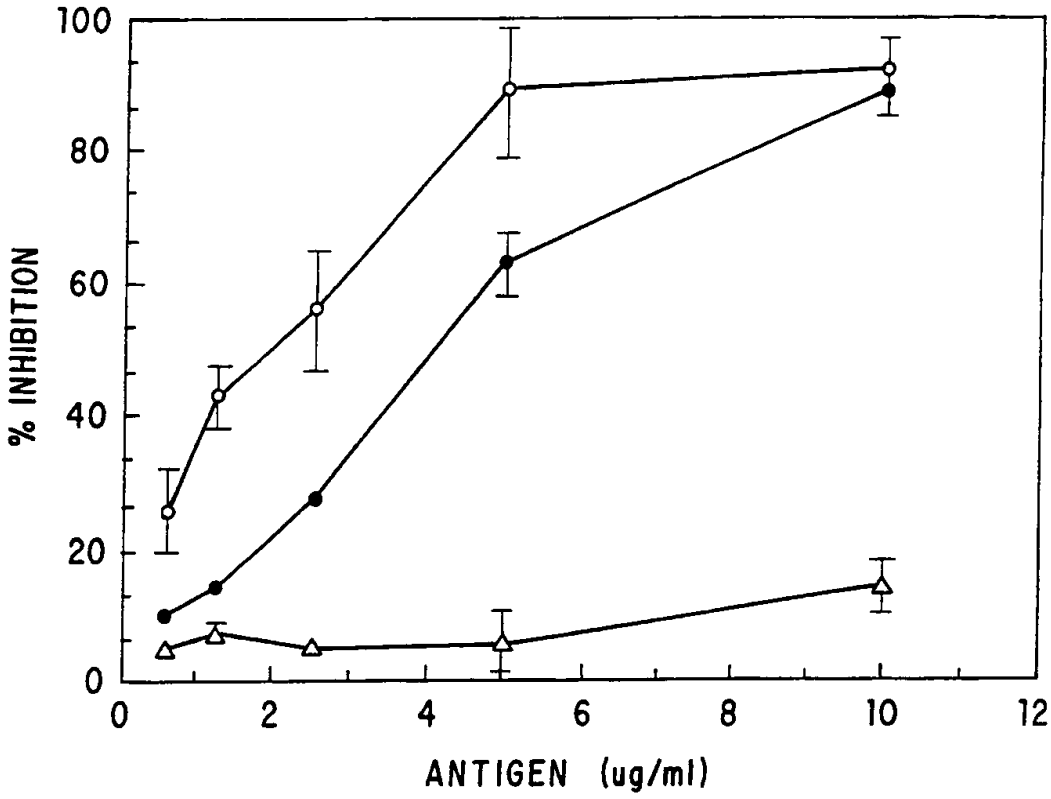

FIG. 3

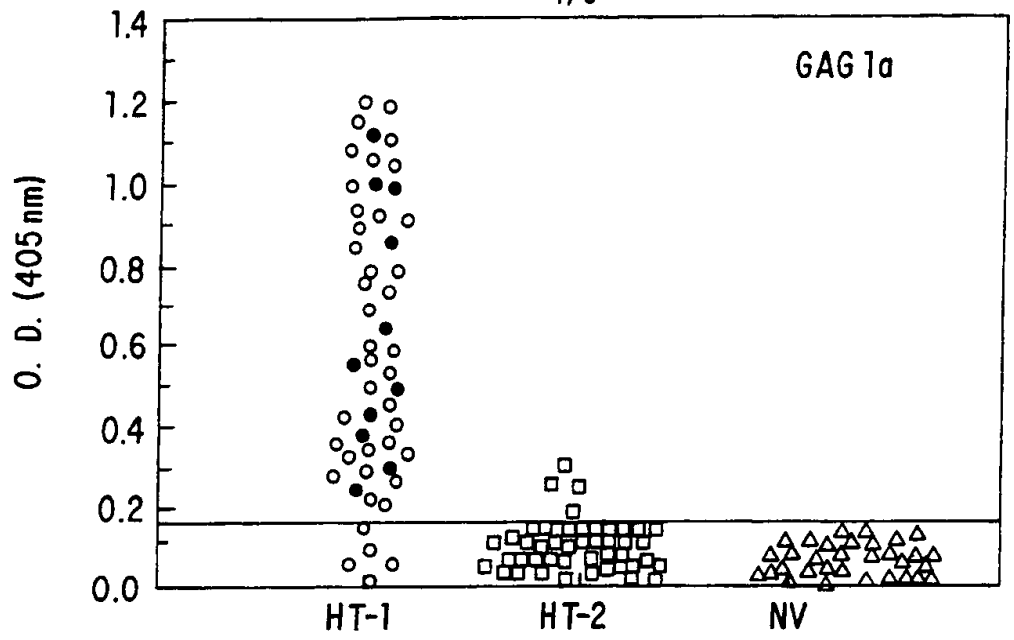
GAG 1a
O. D. (405 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HT-1
HT-2
NV

FIG. 4A

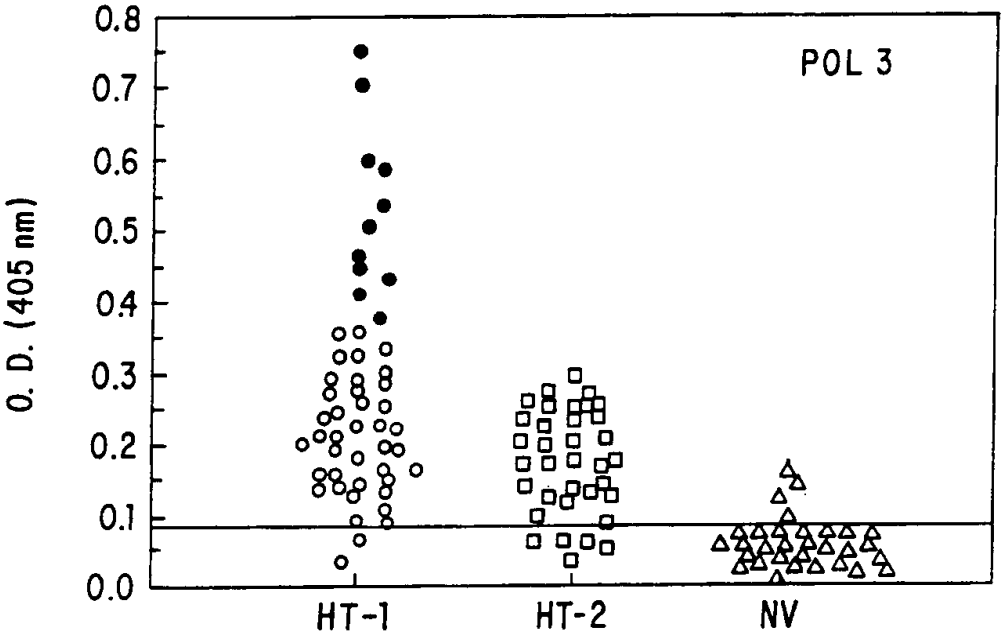
POL 3
O. D. (405 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HT-1
HT-2
NV

FIG. 4B

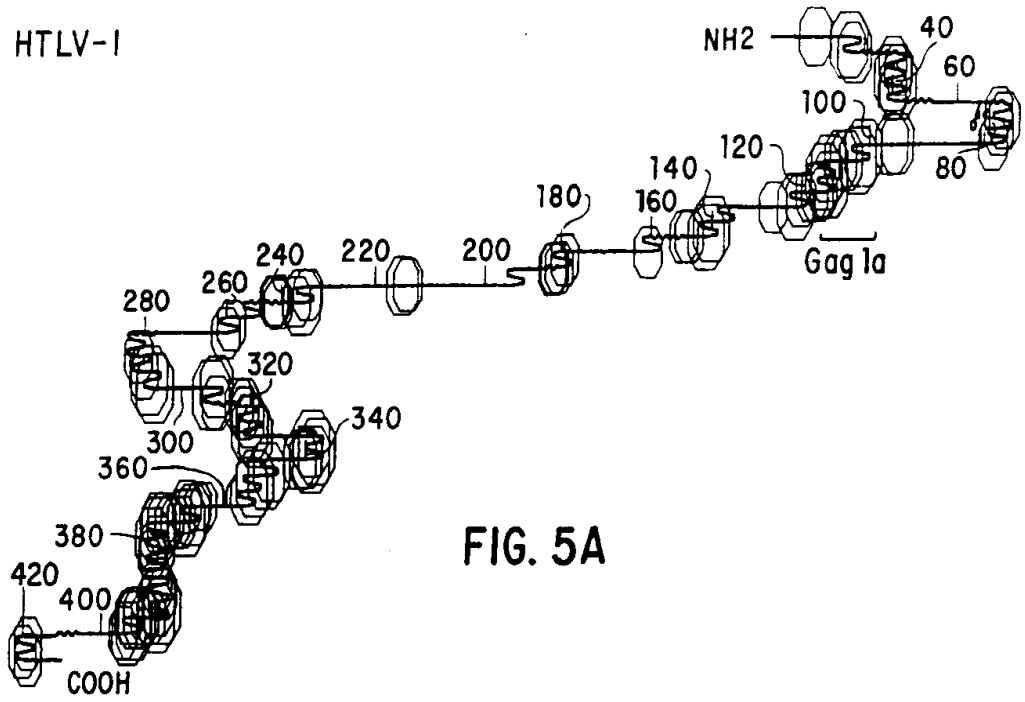
HTLV-I
NH2
40
60
100
120
80
140
160
180
200
220
240
260
280
320
340
300
360
380
420
400
COOH
Gag 1a

FIG. 5A

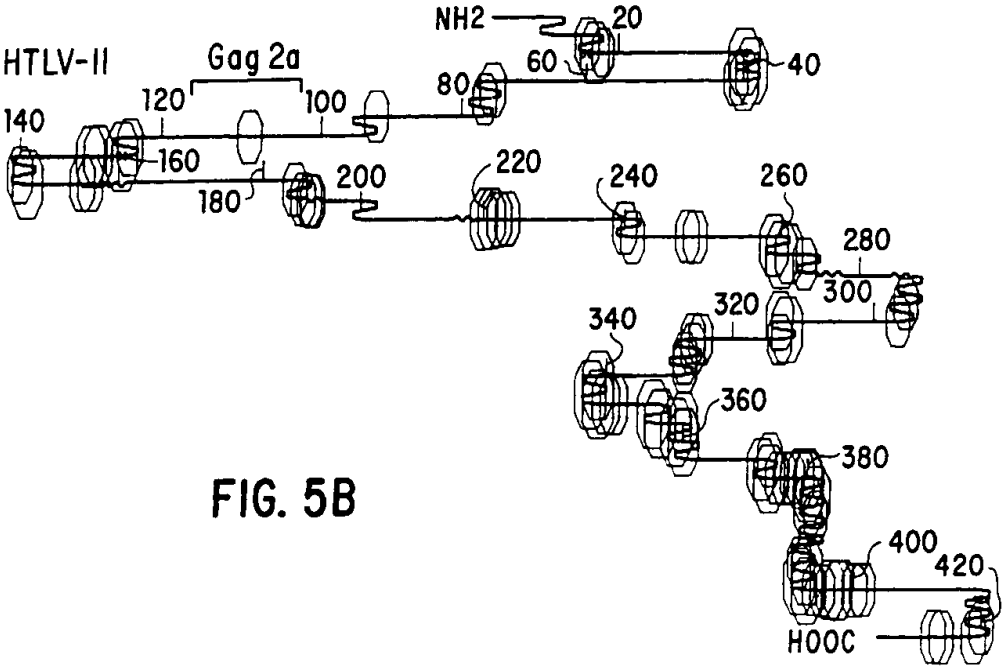
HTLV-II
NH2
20
60
40
80
Gag 2a
120
100
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
HOOC

FIG. 5B

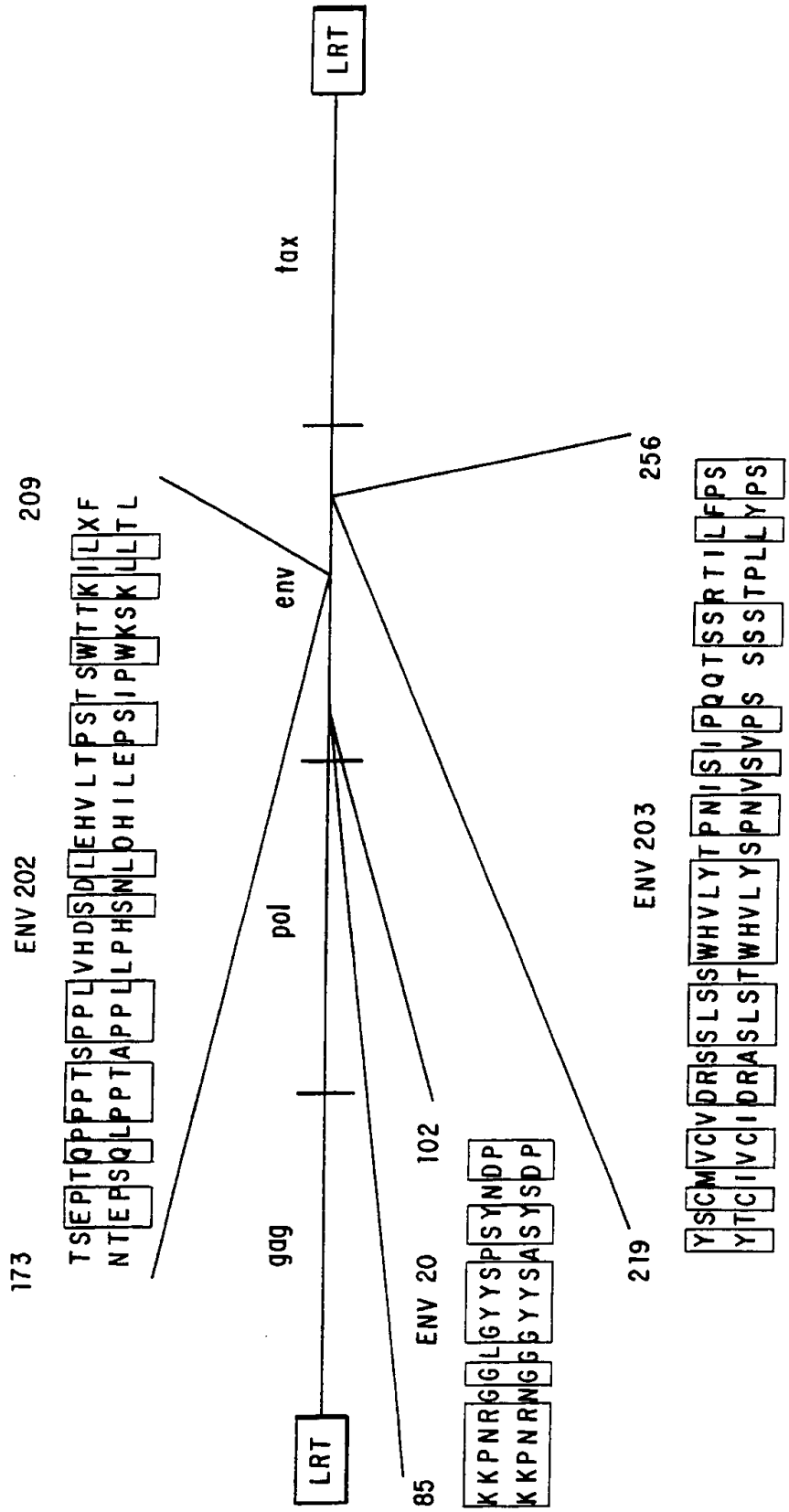
LRT
tax
env
pol
gag
LRT
173
209
ENV 202
85
102
ENV 20
219
256
ENV 203

FIG. 6

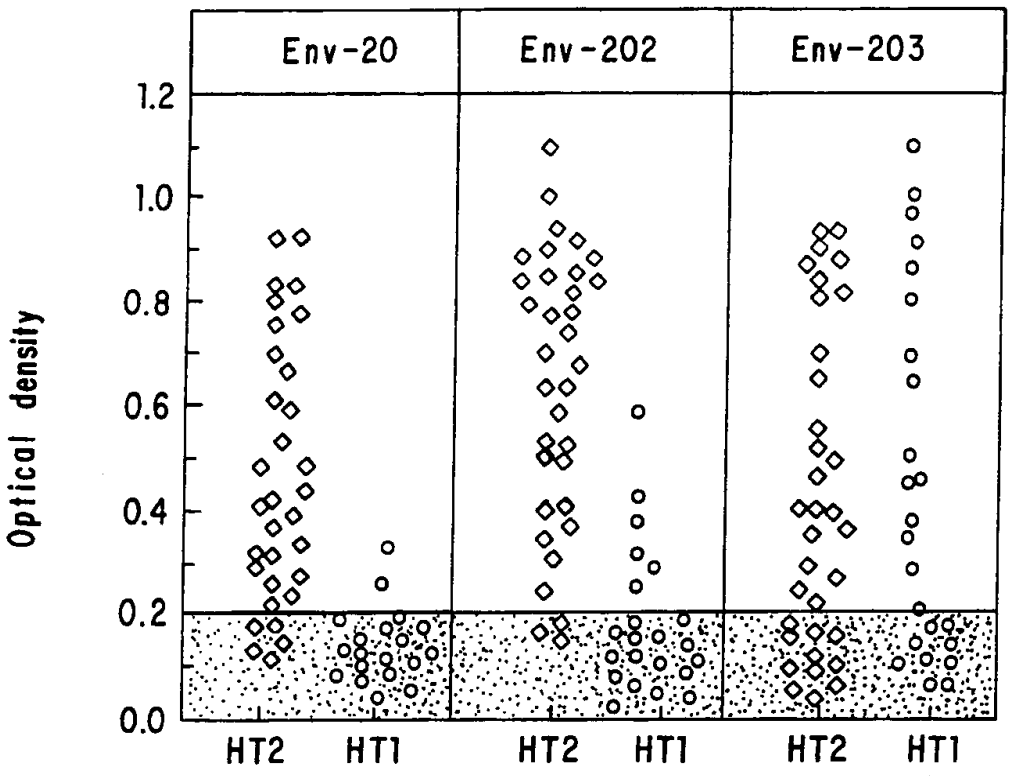
Env-20
Env-202
Env-203
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HT2
HT1
HT2
HT1
HT2
HT1

FIG. 7

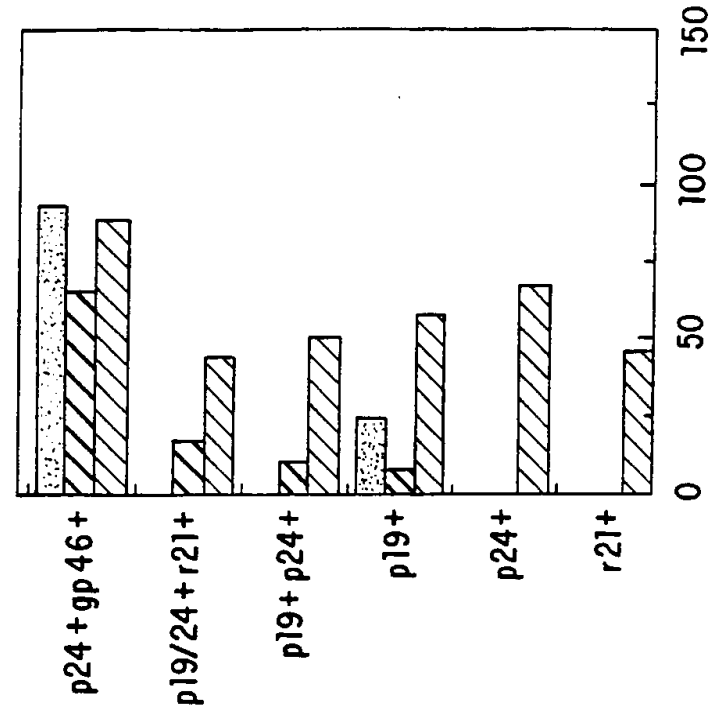
p24 + gp46+
p19/24 + r21+
p19+p24+
p19+
p24+
r21+
0
50
100
150
% positivity

FIG. 8B

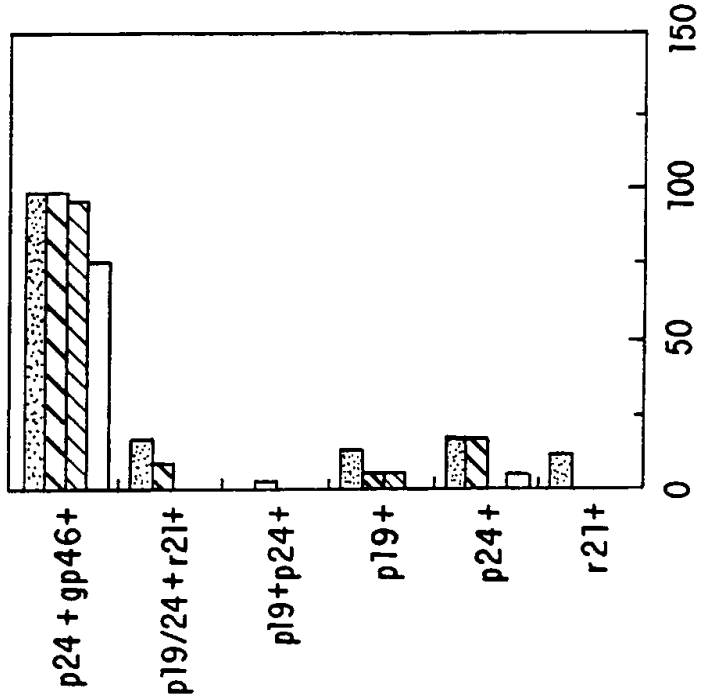
p24 + gp46+
p19/24 + r21+
p19+p24+
p19+
p24+
r21+
0
50
100
150
% positivity

FIG. 8A

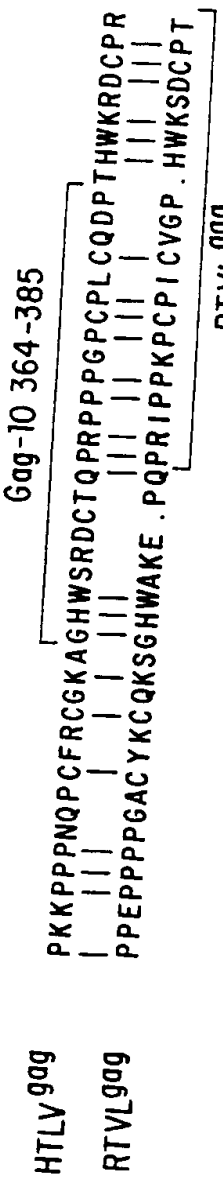
Gag-10 364-385
HTLV gag
RTVL gag
PKKPPPNQPCFRCGKAGHWSRDCTQPRPPPGPCPLCQDPTHWKRDCPR
PPEPPPPGACYKCQKSGHWAKE.PQPRIPPKPCPICVGP.HWKSDCPT
RTVL gag

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06214

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02, 39/12, 37/00; C12Q 1/70

U.S. CL.: 530/325, 326/ 435/5; 424/89; 514/12

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5, 7.92; 436/518, 533, 811, 813, 819; 424/89; 514/12; 530/325,326

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS
STIC Sequence Search

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	WO, A, 90/08162 (Yang) 26 July 1990, see claim 1.	1,2 6,8,11-26
X,P Y	WO, A, 90/10231 (Blomberg et al.) 07 September 1990, see claim 2.	1,3 2,6, 9-26
Y,P	EP, A, 0,424,748 (Buonagurio et al.) 05 February 1991, see Figures 10-13.	1,4,7, 11-26
Y,P	EP, A, 0,423,649 (Maeda et al.), 24 April 1991, see claim 2.	1,5,11-26
Y	WO, A, 89/08664 (Svennerholm et al.) 21 September 1989, see claim 3.	1,8,11-26

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 December 1991	08 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Donna C. Wortman

Form PCT/ISA/210 (second sheet) (Rev. 11-87)